REMARKS

With entry of this amendment, claims 9-10, 15, 18-20 and 32-33 are under examination. Claims 11-14 and 16 have been cancelled by this amendment. Claim 9 has been amended to be limited to leukemia, as supported by Example 9, and to recite "administering" as appropriate to the presently claimed subject matter and in conformance with dependent claim 18. Claim 10 has been amended to conform with the amended language of claim 9. Claim 18 has been amended to add the term "acceptable", for proper grammar. Claims 32 and 33 have been amended to overcome an indefiniteness rejection. Support for the amendments can be found throughout the specification, and in the originally filed claims. No new matter has been added. Reconsideration is requested.

The Examiner objected to the specification because the priority data does not reflect the status of USP 6,777,444. The specification has been amended to include this information. Withdrawal of the objection is respectfully requested.

The Examiner noted that page 34 was missing from the filed application. The amended passages in the previously filed Preliminary Amendment include page 34 from the parent application. It is submitted that addition of the allegedly missing page does not add any new matter, as the parent application was incorporated by reference. It is further confirmed that all of the amendments to the specification made in the preliminary amendment were of a stylistic or formal nature, and did not add any new matter.

Claims 9-12, 14-16, 18-20 and 32-33 were rejected under 35 USC § 112, first paragraph, because the specification allegedly does not provide enablement for the treatment

of all tumor types by administering tetra-O-methylnordihydroguaiaretic acid. The Examiner has cited Bertino and Salmon, "Principles of Cancer Therapy", *Cecil's Textbook of Medicine*, in support of her position. Although Applicants do not agree with the Examiner's position, in order to advance prosecution, the claims have been limited to the treatment of leukemia. As noted by the Examiner, support can be found in Example 9 of the specification. It is respectfully submitted that the present claims are free of the enablement rejection. Reconsideration and withdrawal of the rejection are respectfully requested.

Claims 32-33 were rejected under 35 USC § 112, second paragraph, as being indefinite. The claims have been amended to replace "concentration" with "effective amount" and are believed to be free of the rejection. Reconsideration and withdrawal of the rejection are respectfully requested.

Claims 9-16 and 18-20 were rejected under 35 USC § 102(b) as being anticipated by Neiss et al. (USP 5,276,060). To the extent that this rejection may be considered applicable to the presently pending claims, it is traversed for the following reasons.

Neiss et al. do not disclose the use of the present compounds for the treatment of leukemia, to which the claims are now limited. Reconsideration and withdrawal of the rejection is respectfully requested.

Claims 9-16, 18-20 and 32-33 were rejected under 35 USC § 103(a) as being unpatentable over Neiss et al. To the extent that this rejection may be considered applicable to the presently pending claims, it is traversed for the following reasons.

It is respectfully submitted that there is no suggestion in Neiss et al. that the presently recited compounds could be used to treat leukemia, as presently claimed. Reconsideration and withdrawal of the rejection is respectfully requested.

Claims 9-10, 14-16, 18-20 and 32-33 were rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-8 of USP 6,214,874 and claims 9-10, 14-16, 18-20 and 32-33 over claims 9, 11-14, 17-27 and 41-42 of USP 6,608,108. It is respectfully submitted that this rejection is not applicable to the presently pending claims. Applicants will consider filing a Terminal Disclaimer if the rejection is maintained when otherwise allowable subject matter has been indicated.

The Examiner indicated that certain references in the IDS have not been considered because she was unable to locate copies. Enclosed are copies of the references in question. It is requested that these documents be considered and made of record in the application.

All objections and rejections having been addressed, it is respectfully submitted that the application is in condition for allowance, and Notice to that effect is respectfully requested.

Respectfully submitted,

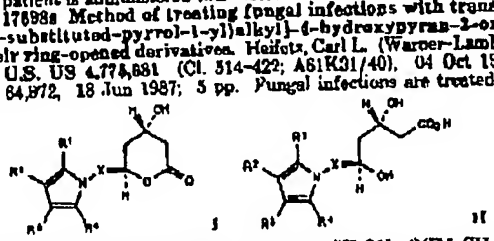
Date: 5/17/07

Ann S. Hobbs, Ph.D.
Registration No. 36,830
VENABLE
P.O. Box 34385
Washington, D.C. 20043-9998
Telephone: (202) 344-4000
Telefax: (202) 344-8300
ashobbs@venable.com

analogs I (B = base; X = S, O where ≥1 X = S; n = 2-30) inhibit the replication of retroviruses and, as such or in the form of block or graft copolymers with normal deoxyribonucleotides, inhibit the replication of neoplastic cells. These polymers are useful for treatment of retroviral infections such as AIDS and of neoplastic diseases. The cytopathic effect of human immunodeficiency virus (HIV) on ATH8 cells was 100% inhibited by I (B = cytosine; X = S; n = 12) at 6 μM. At this concn., the inhibition of the cytopathic effect by I contg. mixed bases increased with increasing guanine plus cytosine content. I having a sequence complementary to the beginning of the *c-myc* oncogene inhibited proliferation and DNA and *c-myc* protein formation by HL-60 leukemia cells at 0.5-30 μM.

111: 17697r **Nucleoside phosphates and their use as antiviral agents for treatment of AIDS and herpes simplex.** Frost, Phillip; Fishman, Jack (Ivax Laboratories, Inc.) Eur. Pat. Appl. EP 244,405 (Cl. C07H19/10). 28 Sep 1988, US Appl. 30,819, 27 Mar 1987; 17 pp. Phosphates $R^1R^2P(O)OH$ [I; R^1, R^2 = 2',3'-dide=oxy-5'-adenosinyl, 3'-azido-3'-deoxy-5'-thymidinyl, 2',3'-dideoxy-=5'-cytidinyl, 2-[(2-amino-1,9-dihydro-6-oxo-6*H*-purin-9-yl)meth=oxy]ethoxy, 2',3'-didehydro-2',3'-dideoxy-3'-cytidinyl], which are useful against herpes simplex infection and AIDS, are prepd. 3'-Azido-3'-deoxythymidine was treated with 2-cyanoethyl phosphate and dicyclohexylcarbodiimide to give the phosphorylated product, which was treated sequentially with 2-mesitylenesulfonyl chloride, 1-methylimidazole and 2',3'-dideoxyadenosine to give I (R^1 = 2',3'-dideoxy-5'-adenosinyl, R^2 = 3'-azido-3'-deoxy-5'-thymidinyl) (II). II was more effective against AIDS virus in vitro than either azidodeoxythymidine, or azidodeoxythymidine and 2',3'-dideoxyadenosine administered as a mixt. II (400 mg) was mixed with CM-cellulose and sucrose and the resultant mixt. was compressed into a tablet; an AIDS patient is administered this tablet 1 time daily.

111: 17698s **Method of treating fungal infections with trans-=6-[(3-substituted-pyrrol-1-yl)alkyl]-4-hydroxypyran-2-ones or their ring-opened derivatives.** Heifetz, Carl L. (Warner-Lambert Co.) U.S. US 4,774,681 (Cl. 514-422; A61K31/40). 04 Oct 1988, Appl. 84,972, 18 Jun 1987; 5 pp. Fungal infections are treated by



I II

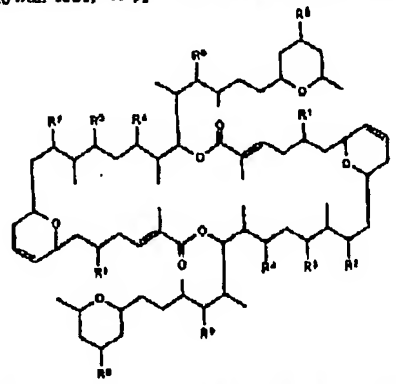
administering the title compds. (I; X = CH_2, CH_2CH_2, $CHMeCH_2$; R^1 = 1- or 2-naphthyl, cyclohexyl, norbornenyl, 2-, 3-, or 4-pyridinyl, Ph, substituted Ph; R^2, R^3 = H, Cl, Br, cyano, CF_3, Ph, C_{1-4} alkyl, C_{2-5} carboalkoxy, CH_2OR^5; R^4 = C_{1-4} alkyl, cyclopropyl, cyclobutyl, CF_3; R^5 = H, C_{2-5} alkanoyl), the corresponding lactone ring-opened 6,δ-dihydroxyheptanoic acid (II; variables same as defined above), or a pharmaceutically acceptable salt thereof. The antifungal activities of 3,4-dibromo-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl=)-1*H*-pyrrole-1-heptanoic acid monosodium salt (III), *trans*-4-=bromo-6-(4-fluorophenyl)-1-[2-(tetrahydro-4-hydroxy-6-oxo-2*H*-=pyran-2-yl)-ethyl]-2-(trifluoromethyl)-1*H*-pyrrole-3-carboxylic acid bis ester, 3,4-dichloro-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-meth=ylethyl)-1*H*-pyrrole-1-heptenoic acid monosodium salt were in vitro tested against *Candida albicans*, *C. tropicans*, *Torulopsis glabrata*, and *Cryptococcus neoformans* using the microbroth dilu. techniques. For example, min. inhibitory concns. for III against 11 strains of *C. albicans* were 16-32 μg/mL; the min. inhibitory concns. of amphotericin B against these strains was 2-8 μg/mL.

111: 17699t **Treatment of helminth infections with zearalene.** Clough, Ellen R.; Edwards, Jii Carl K. (International Minerals and Chemical Corp.) U.S. US 4,778,821 (Cl. 514-460; A61K31/335), 18 Oct 1988, Appl. 78,821, 15 Jul 1987; 7 pp. Helminth infections in animals can be treated with pharmaceuticals contg. ≥1 compds. selected from zearalenone, zearalanone, zearalane (I), zearalenol, zearalanol, or dideoxyzearalane. Mice were administered a compn. contg. I and squalane (carrier) and 24 h later they were infected with *Nippostrongylus brasiliensis* larvae. After 7 days the mice were protected against migrating larvae through the lungs in a dose-dependent manner. I (1000 μg) inhibited 38.1-75.4% of *N. brasiliensis* in mice 1 day after infection; levamisole, in comparison, inhibited 67.1-62.3%.

111: 17700m **Anthracycline antibiotics as anti-retroviral agents.** Kuramochi, Tsuneo (Meiji Seika Kaisha, Ltd.) Eur. Pat. Appl. EP 294,763 (Cl. A61K31/70), 14 Dec 1988, JP Appl. 87/145,538, 10 Jun 1987; 4 pp. Anthracycline antibiotics (e.g. daunorubicin, doxorubicin) show greater antiretroviral activity and less toxicity than conventional agents such as azidothymidine and bleomycin. Prodn. of feline leukemia virus by infected FL74 cells was completely inhibited by exposure of the cells to daunorubicin at 5 μg/mL for 1 h or at 0.05 μg/mL for 6 h. Capsules were prepd. each contg. daunorubicin-HCl 20, lactose 60, and Mg stearate 5 mg.

111: 17701n **Method of treatment of sepsis of newborns.** Chudnov, V. Z.; Sabanshieva, R. U.; Shakhova, O. V. (Scientific-Research Institute of Pediatrics, Academy of Medical Sciences, U.S.S.R.) U.S.S.R. SU 1,438,795 (Cl. A61K39/18), 23 Nov 1988, Appl. 3,821,105, 23 Oct 1984. *From Otkrytiya, Izobret.* 1988, (43), 33. Sepsis in newborns is treated by antibacterial and detoxifying vitamin therapy, and administration of prednisolone and antistaphylococcal γ-globulin. The lethality and treatment periods are shortened by also administering triiodothyronine.

111: 17702p **Antibiotic and antitumor misakinolide compositions and their derivatives.** Higa, Tatsuo; Sakai, Ryuichi Sakai; Lui, May S. (Harbor Branch Oceanographic Institution, Inc.) PCT Int. Appl. WO 88 00,195 (Cl. C07D493/18), 14 Jan 1988, US Appl. 879,134, 26 Jun 1986; 40 pp. Macrolides I (R^1-R^5 = H, OH, alkoxy, acyloxy), derivs. of the *Theonella* (marine sponge) metabolite misakinolide (I; R^1 = R^3 = R^4 = R^5 = OH, R^2 = R^3 = OMe) (II), are prepd. for use as antitumor, antiviral, and antifungal agents. An acetone ext. of *Theonella* was concd. and extd. with H_2OAc to give an oil which, on chromatog. on polystyrene gel and silica gel, yielded II. II produced 50% inhibition of P388 mouse leukemia cells in vitro at 10 ng/mL. II was active against herpes simplex virus 1 and vesicular stomatitis virus in CV-1 cells in vitro at 8 μg/0.5 mL, and showed a min. inhibitory concn. against *Candida albicans* of 6 μg/mL.



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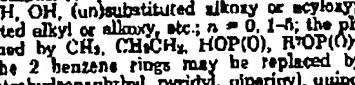
111: 17703q **Peptides comprising the sequence seryl-aspartyl-=lysyl-proline, procedure to extract the tetrapeptide, and applications.** Beerhuyer, Emile; Enriquez, Martine; Chatenu, Maryse; Wdzieczak, Johanna (Institut National de la Sante et de la Recherche Medicale (INSERM); Institut Gustave Roussy) Fr. Demande FR 2,601,678 (Cl. C07K5/10), 22 Jan 1988, Appl. 86/10,486, 18 Jul 1986; 16 pp. The title tetrapeptide (I), derivs. (esp. acetylated or methylated), and peptides of <2000 mol. wt. contg. it are extd. for prepg. medicaments (or protecting bone marrow during chemotherapy of cancer and for prodn. of specific antibodies. Fetal calf bone marrow was homogenized with 10^{-2}M phosphate buffer, pH 7.2, contg. 10^{-4}M mercaptoethanol at 4°, the suspension was centrifuged at 15,000 *g* for 1.5 h, and the supernatant was ultrafiltered (exclusion limit 10^4 daltons), concd., and lyophilized. The material was then chromatographed on Biogel P-2 (200-400 mesh) and eluted with 10^{-2}M HOAc. The active fraction was further purified by chromatog. on Sep-pak C-18 followed by HPLC on ODS-Hypersil C-18. The yield was 60 μg/kg starting material. The product was active in mice at 100 ng/mouse. It inhibited the entry of spleen colony-forming units into DNA synthesis (phase S) induced by Ara-C and enabled animals to survive LDs of Ara-C without inhibiting regression of EMT6 tumors being treated with Ara-C.

111: 17704r **Neoplasm inhibitors comprising metal salts and phenol derivatives.** Jordan, Russell T.; Allen, Larry M. (Chemex Pharmaceuticals, Inc.) PCT Int. Appl. WO 88 03,805 (Cl. A61K33/30). 02 Jun 1988, Appl. 86/U%2547, 19 Nov 1986; 131 pp.



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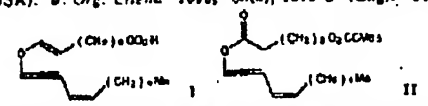
Antitumor compns. comprise a metal salt and the phenols I [D, E, F, X, Y, Z = H, OH, (un)substituted alkoxy or acyloxy; R^1-R^6 = H, (un)substituted alkyl or alkoxy, etc.; n = 0, 1-6; the phenolic groups may be joined by CH_2, CH_2CH_2, HOP(O), $R^7OP(O)$; R^7 = alkyl; either of the 2 benzene rings may be replaced by cyclohexyl, naphthyl, tetrahydronaphthyl, pyridyl, piperinyl, quinolinyl, indanyl or indenyl; any R^4-R^6 may be joined with the benzene carbons to form rings]. The metal salts are of Zn, Cr(III), Y, Co(II), Co(III), Ni, Mg, Al, Cu(I), Cu(II), Fe(III), Cd, Sb, Hg, Rh, V, or rare earth metals. 1-(3,4-Dimethoxyphenyl)-4-(2,3,4-trimethoxyphenyl)butane (prepn. given) was refluxed with HBr under N for 9 h to give 1-(3,4-dihydroxyphenyl)-4-(2,3,4-trihydroxyphenyl)butane (II). Intratumor administration of II together with $ZnCl_2$ enhanced the survival time and decreased tumor incidence in mice with transplanted human breast adenocarcinoma. An ointment contained $ZnCl_2$ 10.0, a catecholic butane 5.0, PEG-400 4.2, PEG-8000 61.7, water 19.0 and ascorbic acid 0.1% by wt.

111: 17705j **Use of 1-(3'-trifluoromethyl-4'-nitrophenyl)-4,4-=dimethylimidazolide-2,5-dione in treatment of hormone-dependent cancers other than that of the prostate.** Mogullewsky, Martine; Bouton, Marie Madeleine (Roussel-UCLAF) PCT Int. Appl. WO 88 03,404 (Cl. A61K3/145), 19 May 1988, FR Appl. 86/15,618, 04 Nov 1986; 12 pp. The title compd. (I) is used to treat hormone-dependent cancers other than that of the prostate or to prep. a medicament to do so. I at 10^{-5}M inhibited the growth of MCF-7 and T47-D tumor cells by 30%.

111: 17706k **Chalcone derivatives useful in controlling growth of tumor tissue and their preparation.** Edwards, Michael L.; Stemerick, David M.; Sunkara, Sai P. (Merrell Dow Pharmaceuticals, Inc.) Eur. Pat. Appl. EP 288,794 (Cl. A61K31/135), 02 Nov 1988, US Appl. 38,227, 09 Apr 1987; 15 pp. Chalcone derivs. I [Ar =

(+) isocarbacyclin (I), based on facile access to (1*S*,2*R*,5*R*)-bicyclo[3.3.0]oct-6-en-*endo*-2-ol has been accomplished, incorporating a controlled ene reaction and elaboration of the ω-appendage via Julia coupling as key steps.

112: 118493x **Total synthesis of colnelenic acid.** Corey, E. J.; Wright, Stephen W. (Dep. Chem., Harvard Univ., Cambridge, MA 02138 USA). *J. Org. Chem.* 1990, 55(5), 1670–3 (Eng). Colnelenic

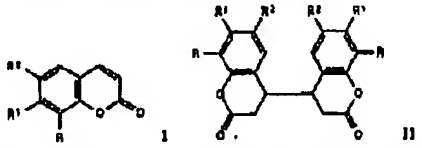


I II

acid (I) has been synthesized from cyclooctene and 1-heptyne. Key steps in the synthesis of I include the formation of the enol ester II and its deoxygenation via its enol phosphate and AlEt₃ under Pd catalysis to form the crit. divinyl ether subunit of I.

112: 118494y **Study of the cobalt(II)-3-hydroxyflavone complex.** Malesev, D.; Radovic, Z. (Inst. Phys. Chem., Pharm. Fak., Belgrade, Yugoslavia). *Pharmazie* 1989, 44(7), 495 (Ger). The title compd. is a 1:1 complex with an absorption max. at 415 nm and a log stability const. of 6.38. It can be used to det. 3-hydroxyflavone at 5–25 × 10⁻⁶ mol L⁻¹ in the absence of interfering cations.

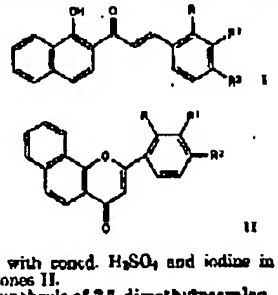
112: 118496z **Reaction of low-valence titanium (titanium tetrachlorides/zinc) with coumarins.** Feng, Juncai; Zhou, Zhaolin; Chen, Weixing (Dep. Chem., Nanjing Univ., Nanjing, Peop. Rep. China). *Huaxue Tongbao* 1989, (6), 37–9 (Ch). Treating



I II

coumarins I (R, R¹, R² = H, Me) with TiCl₄/Zn in THF gave 7.8–83.2% dimers II.

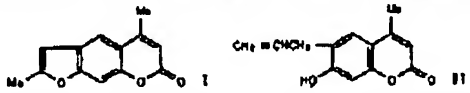
112: 118496a **Synthesis of α-naphthaflavones.** Lu, Yixian; Qin, Huiping; Zhang, Ling; Li, Genxin; Cai, Mengshen (Coll. Pharmacol., Beijing Med. Sch., Beijing, Peop. Rep. China). *Huaxue Tongbao* 1989, (6), 39–41, 36 (Ch). Treating chalcones I (R, R¹, R²



I II

= H, OH, OMe) with concd. H_2SO_4 and iodine in DMSO at 100° gave 35–97% flavones II.

112: 118496b **Synthesis of 2,5-dimethylpsoralen.** Tong, Zengshou; Wu, Kun; Dai, Changshi; Li, Lu (Inst. Radiat. Med., Acad. Mil. Sci., Beijing, Peop. Rep. China). *Zhongguo Yiyao Gongye Zashi* 1989, 20(6), 245–7 (Ch). The synthetic route of 2,5-dimethylpsoralen



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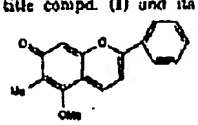
(I) is described. 4-Methyl-6-allyl-7-hydroxycoumarin (II), the key intermediate, was prepd. from resorcinol by 2 different processes.

112: 118498c **Synthesis of dracorhodin.** Gao, Wenfang; Zheng, Hong; Wang, Yushang; Zhang, Zhihong; Lu, Jiangchuan; Zhang, Shoufang (Dep. Org. Chem., Shenyang Coll. Pharm., Shenyang, Peop. Rep. China). *Zhongguo Yiyao Gongye Zashi* 1989, 20(6), 247–50 (Ch). The title compd. (I) and its perchlorate salt were



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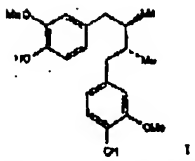
prepd. in 6 steps starting from 2,4,6-(HO)₃C₆H₂CO₂H.

112: 118499d **Regioselective cleavage of the methylenedioxy groups conversion of (-)-austrobailignan-5 to (-)-dihydroguaiaretic acid.** Rao, Koppaka V.; Chattopadhyay, Sunil K. (Coll. Pharm., Univ. Florida, Gainesville, FL 32610 USA). *J. Org. Chem.* 1990, 55(5), 1427–9 (Eng). The methylenedioxy group was selectively cleaved to a methoxyphenol through the use of a *p*-methylthiophenoxide ion. When this procedure was applied to a lignin deriv. such as (-)-austrobailignan-5, the reaction was regioselective, and the intermediate thioacetal could be converted to a catechol, or manipulated to yield either one of the two possible methoxyphenols. Of the two isomers, I was identical with (-)-dihydroguaiaretic acid, which was also isolated for the first time from *Saururus cernuus*.



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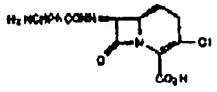
112: 118500x **Synthesis and biological evaluation of 3-chloro-1-carbacepham compounds.** Matsukuma, Ikuo; Yoshiye, Shigeo; Mochida, Kenichi; Hashimoto, Yukio; Sato, Kiyoshi; Okachi, Ryo; Hirata, Tadashi (Tokyo Res. Lab., Kyowa Hakko Kogyo Co., Ltd., Machida, Japan 194). *Chem. Pharm. Bull.* 1989, 37(5), 1239–44 (Eng). The 3-chloro-1-carbacepham nucleus was prepd. for the first



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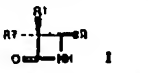
time from a 3*H*-1-carbacepham compd. via addn. of PhSH, oxidn. of sulfide to sulfoxide, α-chlorination of the sulfoxide, and elimination of phenylsulfinic acid. The 2 β-Me analog was similarly prepd., but the 2α-Me analog was not obtained. Optical resoln. of the 3-chloro 1-carbacepham was achieved by using penicillin acylase. Thus, the 7-phenylacetamido deriv. was enantioselectively hydrolyzed to afford the optically active 7-amino-3-chloro-1-carbacepham. Carbacefaclor (I), the carbacepham analog of cefaclor, was directly and efficiently prepd. by enzymic phenylglycylation of the racemic 7-amino-3-chloro-1-carbacepham by using immobilized penicillin acylase. I exhibited comparable antibacterial activity against most gram-pos. bacteria tested and higher activity against typical gram-neg. bacteria than cefaclor. Moreover, I possessed remarkably high chem. stability.

112: 118501y **A novel method for generation of enolizable N-(trimethylsilyl)aldimines and application to β-lactam synthesis.** Uyehara, Tadao; Suzuki, Ichiro; Yamamoto, Yoshinori (Dep. Chem., Tohoku Univ., Sendai, Japan 980). *Tetrahedron Lett.* 1989, 30(32), 4275–8 (Eng). RCH:NSiMe₃ [R = Bu, Me, Ph, CHMeEt,



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CH_2OCH_2Ph, CH_2OMe, $C(OMe):CH_2$] were generated from HCONa $(SiMe_3)_2$ and RLi and were treated with $R^1R^2CHCO_2Et$ (R^1 = R^2 = Me; R^1 = $CHMe_2$, Et, R^2 = H) to give azetidinones I. I (R^1 = Et, R^2 = H) were obtained as a mixt. of isomers.

112: 118502z **Chemistry of O-allylated ketene acetals: a synthesis of β-lactam antibiotics.** Kita, Yasuyuki; Tamura, Osamu; Shibata, Norio; Miki, Takeshi (Fac. Pharm. Sci., Osaka Univ., Suita, Japan 565). *J. Chem. Soc., Perkin Trans. 1* 1989, (10), 1862–4 (Eng). $RNHCOCHR^1CH_2S(O)Ph$ (R = H, CH_2Ph, CHMePh,

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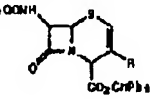
$CHPh_2$; R^1 = H, Me, Et) react with $Me_3OC(CH_2)OSiMe_2CMe_3$ to give the 4-phenylthioazetidin-2-ones I (R^2 = SPh, R^3 = R, $SiMe_2CMe_3$), which are converted into I (R^2 = CH_2CO_2Me), known precursors of various types of carbapenem antibiotics.

112: 118503a **Novel synthesis of C-3 vinylic cephem systems.** Ko, Kwang Youn; Kim, Hongbum; Oh, Jong Hoon; Kim, Moon Hwan; Kim, Wan Joo (Korea Res. Inst. Chem. Technol., Daedeog-Danji, 302-343 S. Korea). *Bull. Korean Chem. Soc.* 1989, 10(4), 363–8 (Eng). The 3-formyl-2-cephem I (R = CHO), available from



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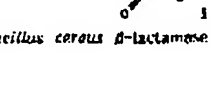
7-aminocephalosporanic acid, has been converted to C-3 vinylic cephems I [R = $CH:CH_2$, (*E*)-CH:CHMe]. The reactions involved are the Grignard addn. to I (R = CHO), the conversion of the resulting alcs. to mesylates, and the elimination of the mesyl group by LiCl.

112: 118504b **The effect of the carboxy group on the chemical and β-lactamase reactivity of β-lactam antibiotics.** Laws, Andrew P.; Page, Michael I. (Dep. Chem. Phys. Sci., Huddersfield Polytech., Huddersfield, UK HD1 3DH). *J. Chem. Soc., Perkin Trans. 2* 1989, (10), 1577–81 (Eng). Kinetic parameters are



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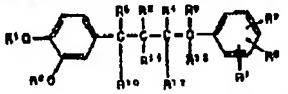
reported for the *Bacillus cereus* β-lactamase I and β-lactamase

(Shiseido Co., Ltd.; Katakura Chikkarin Co., Ltd.) Jpn. Kokai Tokkyo Koho JP 63,119,844 [88,119,844] (Cl. B01J13/00), 24 May 1988, Appl. 86/263,968, 07 Nov 1986; 10 pp. Water-in-oil emulsions useful in pharmaceutical, cosmetic, and food prepns. contain surfactants 2–20, oligosaccharides 1–30, oils 30–90, and H_2O or polyhydric alcs. 1–60% by wt. Chitosan (degree of deacetylation 99%) (15 g) was mixed with 150 mL H_2O and 82.5 mL 1N lactic acid, and the total vol. was adjusted to 300 mL (pH 5.9). This chitosan soln. (10 mL) was incubated 10 min at 37° and treated with 10.5 unit chitosanase for 8 h to give chitosan oligosaccharides. A water-in-oil emulsion contg. 4% by wt. of the chitosan oligosaccharides was prepd.

110: 101816r **Compositions containing catecholic butanes and alan for treating solid tumors.** Jordan, Russell T. (Chemex Pharmaceuticals, Inc.) PCT Int. Appl. WO 88 03,826 (Cl. A61K33/30), 05 May 1988, US Appl. 924,620, 28 Oct 1986; 80 pp.



The title compns., useful for treating benign, premalignant, and malignant tumors, esp. of the skin, contain ≥1 catecholic butane I (R^1, R^2 = H, lower alkyl, lower acyl; R^4–R^8, R^{10}–R^{13} = H, lower alkyl; R^3-R^9 = H, OH, lower alkoxy, lower acyloxy) and ionic Zn. A compn. contg. $ZnCl_2$ 27.5, nordihydroguaiaretic acid 6.9, EDTA 14.7, H_2O 18.8, and poly(ethylene glycol) 32.6% was tested against B-16 melanoma and sarcoma-180 solid tumors grown in mice, by topical application to punctured tumors. Almost all of the tumors demonstrated a significant decrease in size or were completely eliminated.

110: 101817s **Pharmaceutical compositions containing lysine acetylsalicylate for treatment of drepanocytosis.** Benjamin, Lennette (Synthelabo S. A.) Eur. Pat. Appl. EP 274,322 (Cl. A61K31/195), 06 Jul 1988, FR Appl. 86/17,991, 22 Dec 1986; 2 pp. Lysine acetylsalicylate (I) is a drug for the treatment of drepanocytosis. Iv. administration of 3–4 doses of 500 mg I alleviated in humans the swelling and pain assocd. with drepanocytosis. A formulation comprised I 0.9 and glycine 0.1 g. The formulation is dissolved in 5 mL water prior to injection.

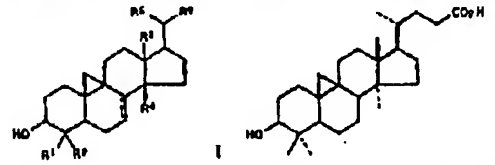
110: 101818t **Implantable biodegradable polymeric drug delivery system with adjuvant activity.** Kohn, Joachim B.; Langer, Robert S.; Nisni, Steven M.; Fox, James G. (Massachusetts Institute of Technology) PCT Int. Appl. WO 88 02,263 (Cl. A61K39/39), 07 Apr 1988, US Appl. 914,380, 02 Oct 1986; 32 pp. An antigen delivery system is described which utilizes a biodegradable polymer with good mech. properties in combination with a material stimulating the immune system. The material having adjuvant activity maybe a polymer degrdn. product or an adjuvant which is contained within or bound to the polymer. The polymer may be formed from tyrosine dipeptides. This polymer is not an adjuvant, but degrades into products which stimulate the immune system. The advantages of this system are that a polymer can be used to form a biodegradable integral structure which is useful as both an implantable source of an antigen or other biol. active compds. and as a control means for the rate of release of the biol. active compd., producing a sustained, relatively const. delivery of antigen with simultaneous stimulation of the immune response. *N*-Cbz-Tyr-Tyr-Hex (CTTH; Cbz = benzyloxycarbonyl; Hex = hexyl) (prepn. given) was treated with CNBr to form the dicyanate, which was mixed with an equimolar quantity of CTTH and the mixt. polymd. in the presence of KOBu-*tert* to give poly(CTTH-iminocarbonate) with mol. wt. 19,500 and which had an intrinsic viscosity 0.27 (DMF, 25°). Using particulate suspensions, the degrdn. products of poly(CTTH-imino-carbonate) were shown to be as potent an adjuvant as complete Freund's adjuvant and muramyl dipeptide when the serum antibody response to bovine serum albumin (BSA) in male CD-1 mice is measured over 56 wk. Further, BSA released from s.c. implanted polymeric antigen delivery devices made of poly(CTTH-iminocarbonate) resulted in significantly higher anti-BSA titers than devices made of poly(bisphenol A-iminocarbonate). At the end of week 56 the injection and implantation sites were examd. and no tissue abnormalities were found, no residues of the injected particulate material were detected, and only small amts. of polymeric residue were detected at the implantation sites.

110: 101819u **Pharmaceuticals containing 7S immunoglobulins, IgG, IgA, or IgM, ceruloplasmin, and transferrin for treatment of mucosa inflammation.** Stuchly, Alfred; Bulik, Josef; Novotna, Lydia; Banda, Imrich; Lucansky, Anton; Andrasim, Jozef Czech. CS 249,228 (Cl. A61K37/04), 18 Mar 1988, Appl. 85/972, 12 Feb 1985; 2 pp. The title formulations, for application to mucosal membranes, contain ceruloplasmin 0.1–0.5, transferrin 0.5–5.0, 7S Ig, IgG, IgA, or IgM 3–30, and coco oil to 1000 wt. parts. A mixt. contg. ceruloplasmin 100; transferrin 500; 7S Ig, IgG, IgA, or IgM 250 mg, and 100 g coco oil was poured into molds forming suppositories weighing 2 g each.

110: 101820v **Sustained-release tablets containing alkali alginates and alkaline earth alginates.** Kiniy, Heinz (Knoll A.-G.) Eur. Pat. Appl. EP 275,338 (Cl. A61K9/22), 27 Jul 1988, Appl. 87/100,672, 20 Jan 1987; 4 pp. Solid pharmaceuticals contain alginates comprising mixts. of an alkali alginate or polyethylene glycol alginate and alkali-alk. earth alginates. These compns. have protracted efficacy. Tablets contained Na alginate 290, Na Ca alginate 30, poly(vinylpyrrolidone) 13, cellulose powder 38, Mg stearate 4, and gallopamil 100 mg each.

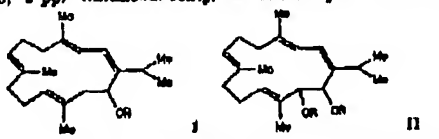
110: 101821p **Antihistaminic pharmaceuticals containing azelastine and theophylline or β-mimetics.** Achterrath-Tuckermann, Uta; Aurich, Rudolf; Engel, Juergen; Hettche, Helmut; Klosmann, Axel (Asta Pharma A.-G.) Ger. Offen. DE 3,743,759 (Cl. A61K31/53), 28 Jul 1988, DE Appl. 3,701,287, 17 Jan 1987; 10 pp. Pharmaceuticals contain azelastine (I) and theophylline (II), or I and ≥1 β-mimetics or their acid addn. salts. The isolated ileum of guinea pigs was incubated in Tyrode soln., suspended in physiol. soln. at 2 g prestress tension; the inhibition of spasms (%) with respect to uninhibited histamine-induced spasms was measured. A combination of 0.001 μg/mL I·HCl and 60 μg/mL II inhibited the spasm effect by 73.1%, whereas 0.001 μg/mL I·HCl or 60 μg/mL II alone inhibited it by 46.3% and 20%, resp. The antihistaminic effect of I increased by 26%, and of II by 24%. A mixt. contg. I·HCl 9, corn starch 18, H_2O 333, and cellulose 300 g was granulated. This granulate was mixed with II·H_2O 900, microcryst. cellulose 180, modified starch (Starch 1500) 88.1, SiO_2 0.9, and Mg stearate 8 g, and tableted. Each 500 mg tablet contained 3 mg I·HCl and 300 mg II·H_2O.

110: 101822q **Steroid derivatives for brain function improvement and formulations containing them.** Seito, Shoji; Matsumoto, Taro; Tokunaga, Yukiko Jpn. Kokai Tokkyo Koho JP 63,145,231 [88,145,231] (Cl. A61K31/575), 17 Jun 1988, Appl. 86/294,867, 10 Dec 1986; 5 pp. The title compds. I (R^1–R^3 = lower alkyl; when R^2



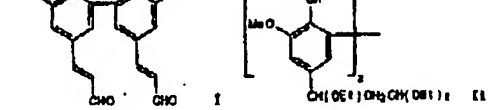
= lower alkenyl, dotted line indicates double bond; when R^3 is carboxyalkyl, dotted line indicates single bond) and their salts are effective as brain performance activators. Capsules contg. steroid II 500 and Mg stearate 10 mg were prepd. EPG indicated that II at 60 mg/kg i.v. activated brain performance in cats.

110: 101823r **Dermatological drugs containing sarcophytol derivatives.** Oda, Koichiro; Hirayama, Koichiro; Umatsu, Kohei (Mitsubishi Kasei Corp.) Jpn. Kokai Tokkyo Koho JP 63,145,223 [88,145,223] (Cl. A61K31/045), 17 Jun 1988, Appl. 86/292,745, 09 Dec 1986; 5 pp. Ointments contg. the title compds. I and II (R^1 =



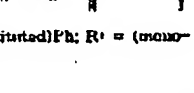
H, acyl) for dermatol. use were prepd. An ointment contg. sarcophytol A 0.1, vaseline 60, paraffin wax 4.9, and cetanol 15% was prepd. A 0.01% soln. of sarcophytol A in Me_2CO inhibited croton oil–induced edema of mouse ear by 47.3%.

110: 101824s **Antiallergy agents containing 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-bis(formylvinyl)biphenyl derivatives and their preparation.** Eda, Shoei; Nishioka, Itsuo; Matsuoka, Yoshiyuki; Chin, Masao (Tsumura Juntendo, Inc.) Jpn. Kokai Tokkyo Koho JP 63,145,225 [88,145,225] (Cl. A61K31/11), 17 Jun 1988, Appl. 86/292,392, 10 Dec 1986; 9 pp. Antiallergy agents



contg. title compds. I (R^1 = R^2 = OH, AcO) are described. Hydrogenolysis of biphenyl compds. II (R = $PhCH_2$) (prepn. given) in EtOH in the presence of 5% Pd/C gave 95% II (R = H), which was refluxed in dioxane and H_2O in the presence of H_3PO_4 to afford 91% (*E*)-I (R^1 = R^2 = OH) (III) and the latter compd. at 100 mg/kg p.o. showed 67.75% control of picryl chloride–induced skin irritation in mice. A capsule was formulated contg. III 6.7, SiO_2 200, and lactose 80 mg.

110: 101825t **Antimycoplasma agents containing pyridone-carboxylic acids.** Nakamura, Shinichi; Sakaguchi, Yuzo; Hashimoto, Shigeki; Abe, Morikazu; Matsumoto, Junichi (Dainippon Pharmaceutical Co., Ltd.) Jpn. Kokai Tokkyo Koho JP 63,152,318 [88,152,318] (Cl. A61K31/47), 24 Jun 1988, JP Appl. 86/186,049, 07 Aug 1986; 9 pp. Antimycoplasma agents contg. title compds. I (R = cyclopropyl,



(mono- or dihalo-substituted)Ph; R^1 = (mono- or di substituted by

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A self-inducing runaway-replication plasmid expression system utilizing the Rop protein

(High-copy-number plasmids; insoluble fusion proteins; helper plasmid; *trp* promoter; *tat* gene; metallothionein; *38K* gene)

Paul E. Giza and Ru Chih C. Huang

Department of Biology, The Johns Hopkins University, Baltimore, MD 21218 (U.S.A.)

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SUMMARY

A highly efficient prokaryotic expression system has been developed that produces proteins at levels exceeding 150 μg/ml of culture medium. The system consists of a temperature-sensitive-copy-number plasmid that carries the *rop* gene and promoter downstream from the *trp* promoter. Any sequence cloned into the *Pvu*II site of the *rop* gene alters Rop protein activity and causes lethal runaway plasmid DNA replication. This plasmid replication can be suppressed in *trans* by complementation with a similar wild-type plasmid. Cells harboring both plasmids are quite stable, and induction of plasmid DNA synthesis occurs only after cells are grown for several generations under conditions that lead to the loss of the *trans*-acting repressor. Large amounts of Rop fusion proteins accumulate in the cell as the *trp* operon is gradually induced via repressor titration. All chimeric proteins accumulate as insoluble aggregates, and are therefore easily purified. They can be solubilized using relatively mild conditions, and the partially purified proteins are highly amenable to cleavage by chemical methods. Using this system we have made Rop fusions with the HIV Tat protein, the herpes simplex virus type-2 38K protein, and Chinese hamster metallothionein.

INTRODUCTION

Rop is a 7.2-kDa, 63-aa plasmid-encoded protein, which acts in concert with RNA I to negatively regulate copy number in some ColE1 and ColE1-like plasmids. The protein was named 'repressor of primer', or Rop, because of its ability to reduce β-galactosidase production to background levels when the *lacZ* gene was placed under the control of the replication primer promoter (Cesareni et al.,

Correspondence to: Dr. R.C.C. Huang, Department of Biology, The Johns Hopkins University, Baltimore, MD 21218 (U.S.A.) Tel. (301)338-7314 Fax (301)338-7733.

Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); Cop^{ts}, temperature-sensitive copy number; dNTPs, the four normal deoxyribonucleoside triphosphates; GuHCl, guanidine hydrochloride; HIV, human immunodeficiency virus (also HTLV-III); HSV, herpes simplex virus; IAA, iodoacetic acid; kb, kilobase(s) or 1000 bp; MT, metallothionein; *MT*, gene coding for MT; nt, nucleotide(s); OD, optical density; *ori*, origin of DNA replication; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^{R}, resistance; Rop, repressor of primer protein; *rop*, gene coding for Rop; SDS, sodium dodecyl sulfate; Tc, tetracycline; Tris, Tris(hydroxymethyl)aminomethane; YT, see MATERIALS AND METHODS, section e; [ ], designates plasmid-carrier state.

1982). Subsequent research has shown that Rop influences plasmid copy number by enhancing or modulating the binding between RNA II, the primer transcript, and RNA I, a small anti-sense transcript of RNA II (Lacatena et al., 1984; Tomizawa, 1986). The binding between RNA I and RNA II leads to transcription termination, thereby preventing DNA synthesis at the *ori*. Because of the modulatory effect of Rop on this RNA–RNA interaction, some researchers refer to this protein as Rom ('RNA one inhibition modulator'; Tomizawa and Som, 1984). For a review of how Rop functions in controlling plasmid copy number, see Davison (1984) and Cesareni and Banner (1985).

We became involved with the Rop protein while attempting to overproduce Chinese hamster MT in bacteria. The *MT* gene was placed under the control of the *trp* promoter and then transferred to a highly amplifiable Cop^{ts} plasmid. We found that cells harboring this plasmid not only produced the MT protein, but also produced another different low M_r protein in large amounts. A computer search of the amino acid sequence identified this additional protein as Rop.

We have developed an expression system that takes advantage of this high level Rop production. This system utilizes a combination of the *rop* promoter and coding sequence, the *trp* promoter, and increased gene dosage via a high-copy-number plasmid. The successful application of this system to the high-level production of HIV Tat protein, HSV-2 38K protein, and Chinese hamster MT is described.

MATERIALS AND METHODS

(a) Materials

Restriction enzymes, T4 DNA ligase, polynucleotide kinase, and PolIk were purchased from New England Biolabs. Restriction enzyme digestions, ligations, and polymerase reactions were performed as recommended by suppliers or as described in Maniatis et al. (1982).

(b) Bacterial strains and plasmids

Escherichia coli K-12 strain HB101 (*pro*, *leu*, *thi*, *lacY*, *hsdR*, *endA*, *recA*, *rpsL*20, *ara*-14, *galK*2, *xyr*-5, *mtl*-1, *supE*44) was used in all transformations. The plasmid pEW2762 was a gift of Drs. E. Wong and B. Polisky. The plasmid pPS21 was a gift of Dr. C. Yanofsky. Plasmid pCV-1 was a gift of Dr. R. Gallo. pCHMTI was a gift of Dr. C.E. Hildebrand. Dr. Laura Aurelian kindly supplied the plasmid pJW7.

(c) Construction of pBR322trp

The plasmid pPS21 is a pBR322 derivative containing the *trp* promoter and *trpE* gene. A 0.5-kb *Hin*fI fragment containing the *trp* promoter and leader gene was isolated on a 6% polyacrylamide gel. This fragment was subjected to a partial *Taq*I digestion, and the resultant fragments were ligated into the *Cla*I site of pBR322. Clones were selected that contained a 232-bp insert (containing the *trp* promoter sequence and the ribosome-binding site) and a regenerated *Cla*I site immediately before the *Hin*dIII site of pBR322. pBR322trp has *trp* promoter transcription in the direction of the Tc^R gene (Sutcliffe, 1979).

(d) Construction of *rop*-fusion plasmids

The fusion pRop-404 was constructed by ligating the 404-bp *Hpa*II fragment from pBR322. The fragment was made blunt-ended by filling-in the CC overhang, and was then ligated to *Pvu*II-digested, phosphatase-treated pPGtrpRopAp.

pRop-MT contains a fusion with the Chinese hamster *MT-I* gene. The starting material for this construction was a 288-bp fragment produced by an *Ava*II + *Hin*fI digestion of the plasmid pCHMTI (Griffith et al., 1983). After ligating *Cla*I linkers the fragment was inserted into pUC9 (Vieira and Messing, 1983) at the *Acc*I site, and both orientations were obtained. The pUC9 clones were restricted with *Eco*RI + *Hin*dIII, and the resultant 326-bp fragments were filled-in and ligated to pPGtrpRopAp.

pRop-Tat contains a fusion with the entire coding sequence of the HIV *trans*-activator, or *tat* gene. To obtain the *tat* gene in a form suitable for fusing to the *rop* gene, pCV-1 (Arya et al., 1985) was digested with *Bam*HI + *Sal*I to obtain a 356-bp fragment. Digestion of this fragment with *Sau*3A produced a 279-bp fragment that encoded the entire *tat* gene except for the first four aa, Met-Glu-

Pro-Val. A synthetic adapter with the sequence 5'-CTGCATGGAGCCAGTA / GACGTACCTCGGTCATCTAG was made that encoded Cys-Met-Glu-Pro-Val and supplied aa 52 of Rop (Cys) as well as the missing four aa of Tat. The unphosphorylated adapter was ligated to the 279-bp *Sau*3A fragment to form a 315-bp blunt-end fragment which was phosphorylated, and ligated to the expression vector.

pRop-Herpes-38K encodes a fusion protein with the HSV type-2 38K protein. The plasmid pJW7 contains the entire coding region of the 38K protein which was excised from the plasmid by digestion with *Nco*I + *Xba*I. The 1.0-kb fragment was isolated, filled in with PolIk and dNTPs, and ligated to the expression vector.

(e) Transformations and cultivation of cells transformed with expression plasmids

HB101 was made competent for transformation with pPGtrpRopAp and pPGtrpRopTc by the standard $CaCl_2$ procedure (Maniatis et al., 1982), except that cells were cultivated at 30°C after the heat-shock treatment. Ap and Tc were used at concentrations of 100 μg/ml and 10 μg/ml, respectively. YT medium (Miller, 1972) consisted of 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose.

The host HB101 [pPGtrpRopTc] is HB101 transformed with the vector pPGtrpRopTc, the helper plasmid which was used for the isolation of plasmids containing fragments inserted into the *Pvu*II site of pPGtrpRopAp. A 100-fold dilution of fresh overnight culture of HB101[pPGtrpRopTc] (grown at 30°C in 10 μg Tc/ml) was made in YT medium (5 μg Tc/ml), grown to mid-log phase, made competent, and transformed in the same manner as HB101. After the heat shock the cells were diluted with 100 volumes of YT medium and incubated at 30°C for 30 min; Tc was added to a concentration of 7 μg/ml. After an additional 2 h of incubation, Ap was added to a concentration of 100 μg/ml, and incubation was continued until saturation was achieved. After appropriate dilutions were made, cells were plated on YT plates (100 μg Ap/ml, 7 μg Tc/ml) and incubated at 30°C to obtain individual colonies. Colonies were inoculated in 2 ml YT medium containing the same antibiotics and the DNA from these doubly transformed cells was extracted (Birnboim and Doly, 1979) and analyzed to determine the proper structure. Double transformants were stored in glycerol at −20°C and used to make fresh starter cultures for fusion protein production.

(f) Protein quantitation and amino acid sequencing

0.1% SDS–15.5% polyacrylamide gels (Laemmli, 1970) were stained (0.2% Coomassie Brilliant Blue/50% methanol/12% glacial acetic acid), destained, and scanned using a Kontes Model 800 Fiber Optic Scanner; peaks were quantitated using a Hewlett Packard 3390 A Reporting Integrator. The Rop protein was sequenced by Applied Biosystems (Foster City, CA) after purification of the protein by hydroxyapatite chromatography. Fragments obtained from chemical cleavage reactions were separated on the same gels, electroblotted, and sequenced as described by Hsieh et al. (1988).

RESULTS AND DISCUSSION

(a) Construction of the *rop* expression vectors

The steps involved in the construction of the two Rop overproducers are detailed in Fig. 1. The 7.3-kb plasmid, pEW2762 (Wong et al., 1982), was the source of the Cop^{ts} phenotype of both Rop protein overproducers. This plasmid contains two mutations in stem-loop IV of the primer transcript, or RNA II, that cause elevated copy number when cells harboring the plasmid are grown at 42°C (Wong et al., 1982; Wong and Polisky, 1985). RNA I is thought to exert its negative control on copy number through the interaction its stem-loop structures with the stem-loop structures of the primer transcript (Davison, 1984; Cesareni and Banner, 1985; Wong and Polisky, 1985; Tomizawa, 1986).

We first noticed the overproduction of the Rop protein when the *trp* promoter was cloned upstream from the *rop* gene and promoter, which were located on pEW2762. To eliminate nonessential DNA and reduce multiple restriction sites, a smaller version of pEW2762 was made (pPGRop) which contained a single *Pvu*II site in the *rop*-coding sequence. The *ori* and copy-number control elements of pPGRop were combined with the *trp* promoter elements of pBR322trp to produce pPGtrpRopA. This combi-

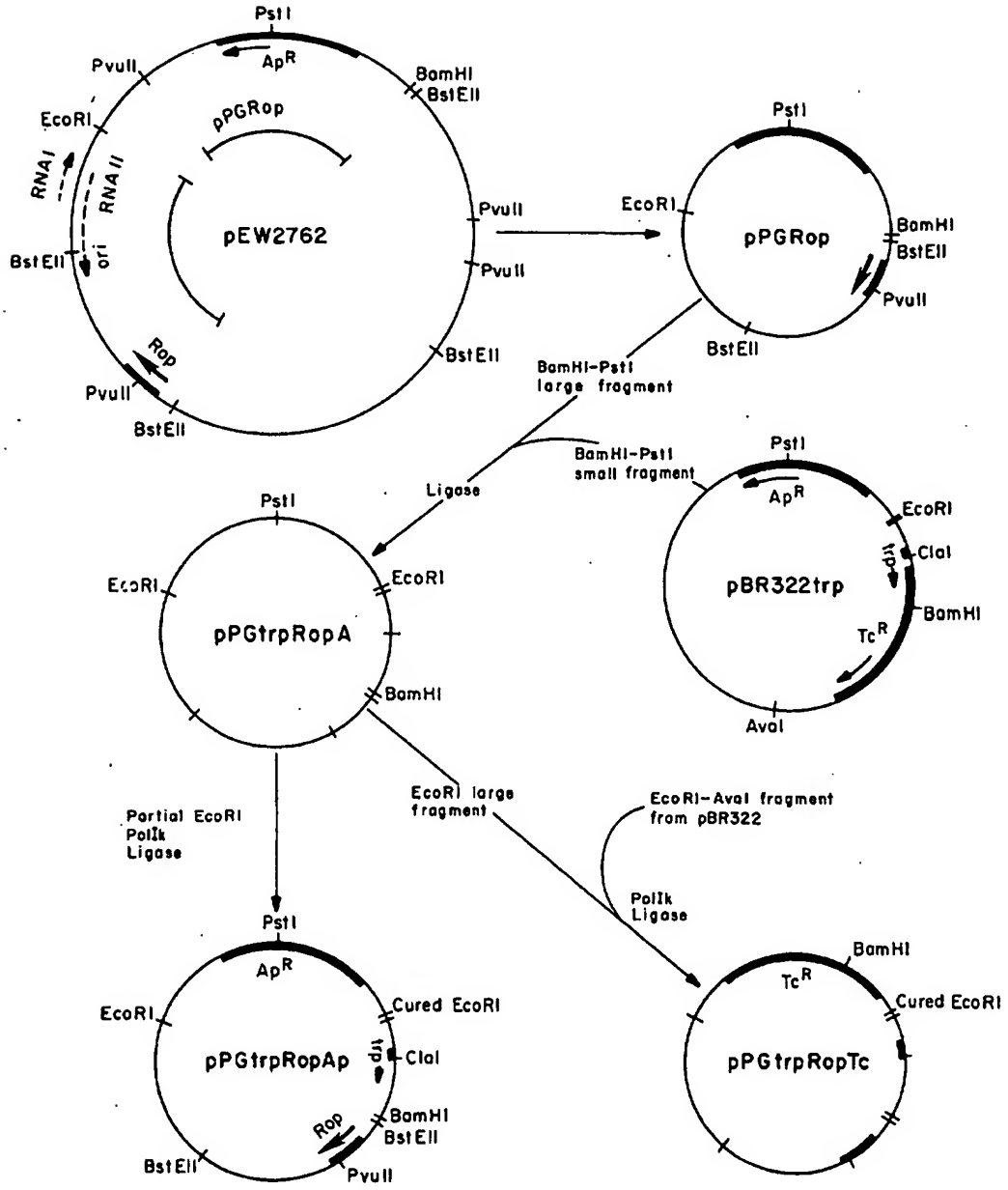

Fig. 1. Construction of expression vectors pPGtrpRopTc (helper plasmid) and pPGtrpRopAp. pEW2762 was digested with *Bst*EII and a 1.1-kb fragment (containing the *rop* gene and *ori*) and 3.0-kb fragment (containing the regions coding for RNA I, RNA II, and Ap^R, were isolated and ligated to form a 4.1-kb plasmid (pEW2762dl) containing two *Bst*EII and two *Pvu*II sites (structure not shown). Cleavage of this plasmid with *Pvu*II + *Eco*RI, followed by blunt-end ligation (*Eco*RI ends made blunt with PolIk and dNTPs) of the two larger fragments yielded pPGRop (3.7-kb). pPGRop was digested with *Pst*I + *Bam*HI, and the larger fragment (2.8-kb) was ligated to the smaller *Pst*I-*Bam*HI fragment (1.4-kb) of pBR322trp (construction described in MATERIALS AND METHODS, section c) to produce pPGtrpRopA. The expression vector pPGtrpRopAp was produced by elimination of the *Eco*RI site near the *trp* promoter, to facilitate future constructions. The plasmid pPGtrpRopTc is identical to pPGtrpRopAp except that the *Eco*RI-*Ava*I fragment of pBR322 that confers Tc^R was ligated between the *Eco*RI sites of pPGtrpRopA. Heavy lines represent the coding regions for Rop, Ap^R and Tc^R. The small box at the *Cla*I site in pBR322trp represents the *trp* promoter sequence. Restriction sites important to plasmid construction are shown; some sites are not labeled but are represented by a bar. Arrows denote the direction of transcription of the various genes. Dashed arrows denote the direction of transcription of RNA I and RNA II. *ori*, the origin of DNA replication.

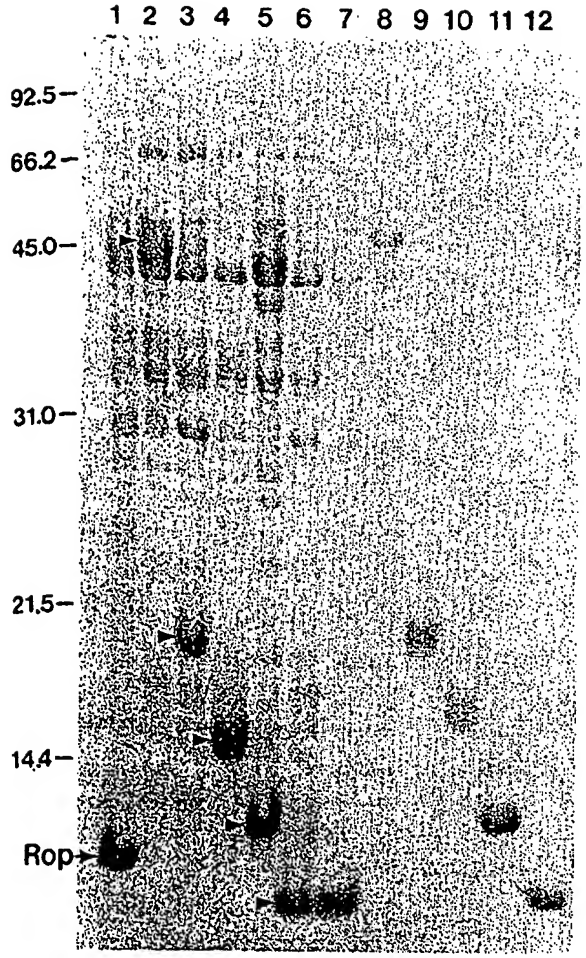

Fig. 2. 0.1% SDS/16% polyacrylamide gel of overproduced proteins. Lane 1 contains cells from Rop overproducer HB101 [pPGtrpRopAp] after induction in M9 medium (proteins from 15 μl of culture). A 1000-fold dilution of an overnight culture (grown at 30°C in YT medium containing 100 μg Ap/ml) was made in 1 liter YT medium (300 μg Ap/ml) and incubated at 39°C for 18 h. Cells were pelleted and resuspended in 1 liter of M9 medium (Miller, 1972) which was supplemented with 0.4% casamino acids, 50 μg 3β-indolylacrylic acid/ml, 300 μg Ap/ml, and induced for 18 h at 30°C. After pelleting and washing, the cells were subjected to a periplasmic extraction as described by Neu and Heppel (1965). Lane 7 represents the periplasmic extracted proteins from the same number of cells as lane 1. Lanes 2–6 contain total SDS soluble protein (25–30 μl of culture) after induction for 40 h at 30°C. The inductions are arranged by decreasing M_r of the fusion proteins in the order Rop-HSV 38K, Rop-Tat, Rop-MT, Rop-404, and Rop-MT(opp). Induction growth was initiated by inoculating 1 μl of starter cultures (see MATERIALS AND METHODS, section e) in 50 ml YT medium (500 μg Ap/ml). After incubation for 40 h at 30°C, cells were pelleted, washed in 20 mM Tris (pH 7.8), lysozyme treated (10^{10} cells/ml), sonicated, and insoluble fusion proteins recovered as described by Kaplan and Greenberg (1987). Lanes 8–12 contain GuHCl solubilized fusion proteins from the same number of cells as represented in lanes 2–6. Prior to electrophoresis the GuHCl was removed by dialysis against water. After lyophilization the proteins were dissolved in sample loading buffer (Laemmli, 1970). All lanes contain proteins from 2×10^7 cells. Gel was stained as described in MATERIALS AND METHODS, section f.

nation of fragments placed the *trp* promoter about 500 bp upstream from the *rop* promoter and coding sequence. The plasmid pPGtrpRopAp (4.1 kb) was the vector used for all gene fusion experiments. It contains a single *Pvu*II site, confers Ap resistance, and displays runaway plasmid replication when grown at temperatures above 37°C. The plasmid pPGtrpRopTc (3.9 kb) confers Tc resistance and has the same temperature-dependent copy number as pPGtrpRopAp. The Tc^R plasmid is integral to the functioning of this system because it supplies Rop activity to cells transformed with lethal runaway copy number *rop* fusion plasmids.

(b) Rop protein overproduction in the strain HB101[pPGtrpRopAp]

Maximum production of Rop protein was achieved by first growing the cultures overnight at 39–40°C in YT medium. Under these conditions the *trp* promoter is partially repressed and cellular plasmid DNA levels reach 15–20 μg/ml. After centrifugation and washing, the cells were resuspended in M9 medium containing 3β-indolylacrylic acid and incubated at 30°C. This second incubation was under conditions that favored total derepression of the *trp* promoter but did not favor runaway plasmid replication.

Fig. 2 illustrates the results of this two step procedure when applied to cells harboring pPGtrpRopAp. After plasmid amplification at 39°C and protein induction at 30°C, Rop protein accumulated to 31% of the bacterial protein shown in lane 1. Even though Rop is not post-translationally modified, more than 85% of the Rop protein was obtained by a simple periplasmic extraction of the pelleted cells. The Rop protein obtained in the periplasmic extract (lane 7) was determined to be 81% pure with less than 5% of the protein remaining in the cell pellet (not shown) after a single extraction.

An interesting feature of this protein production system is that Rop is practically the only protein labeled if the isotope is added after overnight induc-

tion in M9 medium. This selective labeling was quite useful because the entire labeling reaction could be used as a radioactive marker in large scale purifications or other manipulations.

The weight of cells processed from this induction was 7.8 g or about 1 liter of culture at an OD_{660} of 4.4. Periplasmic extracts shown in lane 7 gave values of 190 μg/ml using the Bio-Rad protein assay (standard No. 2) and 300 μg/ml using gravimetric methods. Since Rop constituted about 80% of the extract, the yields were 150 μg/ml and 240 μg/ml, respectively. Quantitative sequencing data showed better correlation to the 240 μg/ml estimate, suggesting that Bio-Rad Standard No. 1 is a more appropriate standard for the quantitation of purified Rop samples.

(c) Construction of *rop* fusion plasmids

The *rop* gene contains a *Pvu*II restriction site that cleaves the coding sequence after the 2nd nt of codon No. 51 (AG/C), 12 aa from the C terminus. DNA fragments can be blunt-end ligated at this point to encode Rop fusion proteins beginning at aa 52. The aa 51 is maintained as a Ser or converted to an Arg depending upon the first nucleotide of the ligated fragment. This is illustrated in Fig. 3 which shows the DNA and amino acid sequences around the *Pvu*II site.

The fusion plasmid pRop-404 was constructed to serve as the initial test of the system. This construct re-established the Ser codon at aa 51 and added an additional 51 aa before encountering a stop codon. The construct pRop-MT consists of a ligation of a pUC9 fragment containing the entire *MT* gene. The fusion Rop-MT(opp) is the opposite orientation of the *MT* gene in the pUC9 fragment. Both constructs produce a Ser-to-Arg mutation at aa 51 (recreating the pUC9 *Eco*RI site), and contain the pUC9 polylinker sequence encoding the six aa from aa 52 to aa 57. The pRop-MT plasmid encodes the entire MT protein beginning at aa 58 and ending at aa 118. The pRop-MT(opp) construct encodes a protein of 73 aa (8.5 kDa); because double stop codons are encountered 22 aa from the fusion point.

Despite repeated attempts, no fusion transformants could be obtained when HB101 was transformed with ligation reactions that interrupted the *rop* gene. Cells harboring pRop-404 produced colonies at 30°C on Ap plates, but colony inoculations

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      Rop protein:  63 aa; 7.2 kDa
     ┌PvuII
CGCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTC
ArgSerCysLeuAlaArgPheGlyAspAspGlyGluAsnLeu
50              55              60


CGCAGCGGTGAGCGTGGGTCTCGCGGTATCATT     pRop-404
ArgSerGlyGluArgGlySerArgGlyIleIle     102; 11.4
50              55              60


CGCAGAATTCCCGGGGATCCGTCGATGGACCCC     pRop-MT
ArgArgIleProGlyAspProSerMetAspPro     118; 12.6
50              55              60


CGCAGAATTCCCGGGGATCCGTCGATGAATCAC     pRop-MT(opp)
ArgArgIleProGlyAspProSerMetAsnHis     73; 8.5
50              55              60


CGCAGCTGCATGGAGCCAGTAGATCCTAGACTA     pRop-Tat
ArgSerCysMetGluProValAspProArgLeu     138; 15.8
50              55              60


CGCAGCATGGATCCCGCCGTCTCCCCCGCGAGC     pRop-HSV 38K
ArgSerMetAspProAlaValSerProAlaSer     388; 43.6
50              55              60
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Fig. 3. The nucleotide and amino acid sequences at the fusion points. The last 14 aa of the Rop protein (Cesareni et al., 1982) and the 11-aa region surrounding the points of fusion are shown. The underlined portions of the aa sequences denote the extent of uninterrupted Rop sequence, and the arrows above the nt sequences mark the points of ligation. The numbers beneath the fusion designations denote the number of aa in the protein and the calculated size in kDa based upon the reading frame established at the point of ligation. The Met's at aa 58 in pRop-MT, aa 53 in pRop-Tat, and aa 52 in pRop-HSV 38K mark the beginning of the aa sequence of the native protein.

in YT medium yielded cultures that produced barely detectable growth after 48 h at 30°C. No colonies were obtained with either *MT* ligation. The same results were obtained when the fragments were ligated to pPGRop. Since this vector does not contain the *trp* promoter, protein overproduction was probably not the reason for growth inhibition. We suspected that the combination of Rop inactivation and the replication primer mutations caused lethal, unconditional plasmid replication.

We constructed the vector pPGtrpRopTc to see if the presumed runaway replication of the plasmid

could be suppressed by supplying functional Rop protein activity to cells harboring these fusion plasmids. This strategy was used by several investigators to identify factors, carried on co-resident plasmids, that suppressed certain runaway-copy-number mutations (Shepard et al., 1979; Twigg and Sherrat, 1980). When the host HB101[pPGtrpRopTc] was transformed with the fusion ligation reactions (MATERIALS AND METHODS, section e) and plated on YT agar containing both Ap and Tc, colonies were obtained after 18–24 h at 30°C. Plasmid DNA extracted from cultures of doubly transformed cells (grown in both antibiotics at 30°C) showed both plasmid species in about equal amounts and at normal levels (approximately 500 ng/ml). Small amounts of the fusion plasmids were isolated on agarose gels in order to eliminate the helper plasmid. No transformants could be obtained when using this DNA to transform HB101. This result confirmed the lethality of these constructs and illustrated the necessity of using the *trans* helper plasmid.

(d) Production of fusion proteins using doubly transformed cells

After several unsuccessful attempts at producing plasmid amplification by shifting the culture temperature, we found that simply diluting doubly transformed cells in YT medium (30°C) containing only Ap resulted in the gradual loss of the helper plasmid. The *trp* operator was gradually induced via repressor titration as fusion plasmid DNA levels increased, and fusion proteins accumulated to high levels.

Fig. 2, lanes 4, 5, and 6 show the results of the growth and induction of cells harboring pRop-MT, pRop-404, and pRop-MT(opp), respectively. The prominent protein bands (arrows) show good agreement with the predicted M_rs for these proteins, and the average accumulation was 20% of the total bacterial protein after 40 h of growth at 30°C. Rop-MT(opp) appears to be about the same size as the Rop protein in lane 7; however, its higher M_r was obvious on peptide mapping gels (Jue and Doolittle, 1984).

To confirm the general applicability of this protein production system, two additional constructs were undertaken. Fusions were made with the HIV Tat protein and the HSV type-2 38K protein. The Tat protein was chosen because of its importance in the study of the HIV disease process (Aldovini et al., 1986; Muesing et al., 1987) and because of its similarities to the MTs (Frankel et al., 1988). The HSV protein was chosen because of its sequence homology to the small subunit of ribonucleotide reductase of several pathogenic viruses (Gibson et al., 1984; Swain and Galloway, 1986). We also wanted to determine if there was a size limitation to the high level protein production.

Fig. 2, lanes 2 and 3, shows the result of the growth and induction of cells harboring pRop-HSV 38K and pRop-Tat, respectively. Prominent bands of the proper M_r (arrows) are obvious in these lanes, and the accumulations of both were about 20% of the total bacterial protein.

(e) Protein purification

Purification of the fusions was necessary in order to confirm their identity by peptide mapping, because antibodies to most of the proteins were not readily available. To our surprise all of the fusions accumulated as insoluble aggregates and were easily purified. Fig. 2, lanes 8–12, shows the partially purified proteins after sonicated cells were fractionated by centrifugation and dissolved in 6 M GuHCl. Densitometric scans of the gel indicated that the fusions accounted for 65% of the protein (75–80% with additional pellet washings) in the extracts, and the average yield was 105 μg/ml of growth medium. All the fusions could also be dissolved at concentrations of 5–10 mg/ml in 1–5 M urea or 1% SDS. These protein stocks could be diluted to 1 M urea without precipitation of the protein. We have prepared stocks of Rop-Tat, free of denaturing agents, in 10 mM Tris (pH 7.0), 1 M NaCl. The Rop-Tat and Rop-MT GuHCl stocks are dark amber in color, whereas the other extracts are colorless. This coloration may be due to the metal-binding properties of these two proteins.

(f) Protein identification by chemical cleavage

The five fusions produced by this expression system were identified by their fragmentation patterns after cleavage at Met with CNBr, and/or at acid-labile Asp-Pro bonds. Fig. 4 shows the results of some of these reactions. The dipeptide sequence Asp-Pro occurs twice in the Rop-HSV 38K protein

(at aa 53–54 and 62–63), and cleavage at these positions should yield high M_r fragments of about 36 kDa. The doublet in lane 2 (arrow) corresponds to these two fragments generated by this partial cleavage reaction. Cleavage of the full size fusion protein at Met (at six positions) should produce several fragments. The largest fragment, beginning at aa 53 (Fig. 3) is the 179-aa, 20.2-kDa band marked by the arrow in Fig. 4, lane 3. This protein was sequenced through the first 16 aa to confirm the identity of the fusion. The rest of the bands clustered below 10 kDa and were not resolved into individual bands on this gel.

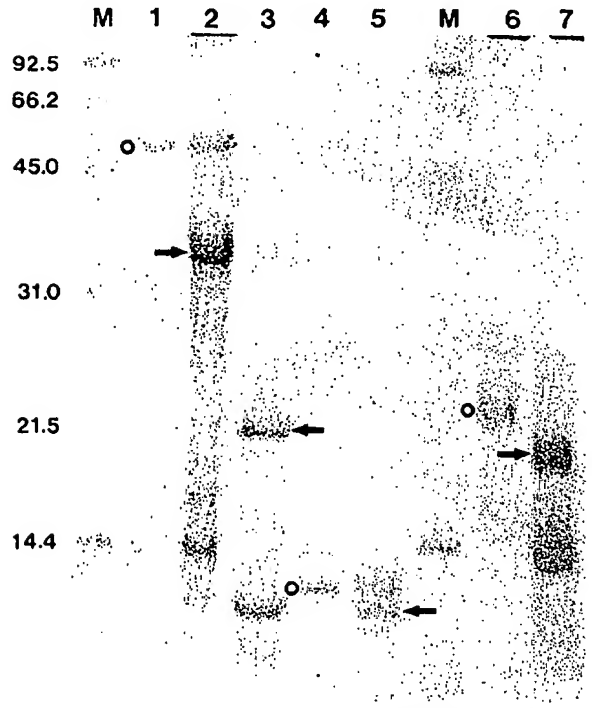

Fig. 4. Chemical cleavage of partially purified fusion proteins. Lanes: 1, Rop-HSV 38K GuHCl extract; 2, Rop-HSV 38K cleaved at Asp-Pro by incubation for 24 h in 50% formic acid/6 M GuHCl at 40°C (Szoka et al., 1986); 3, Rop-HSV 38K cleaved at Met by incubation for 24 h in 70% formic acid/4 mg CNBr/ml at 24°C (Jue and Doolittle, 1984); 4, Rop-404 GuHCl extract; 5, Rop-404 cleaved at Met under same conditions as lane 3; 6, Rop-Tat GuHCl extract; 7, Rop-Tat cleaved at Met under same conditions as lane 3. All of the cleavage reactions were performed on GuHCl solubilized proteins after dialysis of the stocks against water and lyophilization. The dried proteins were dissolved in formic acid or formic acid/GuHCl at concentrations ranging from 1–5 mg/ml. The CNBr reactions were lyophilized, and portions were dissolved in sample loading buffer (Laemmli, 1970). Cleavage reactions at Asp-Pro were dialyzed (4°C) against several changes of 20 mM Tris (pH 8.0) and finally against water. After lyophilization portions were dissolved in sample loading buffer. The gel (0.1% SDS/16% polyacrylamide) was stained as described in MATERIALS AND METHODS, section f. The full size proteins (lanes 1, 4, 6) are marked by circles, and the cleavage products discussed in RESULTS AND DISCUSSION, section f, are marked by arrows. For the complete amino acid sequence of these proteins see Gibson et al. (1984) (HSV 38K protein); Aldovini et al. (1986) (Tat protein); Griffith et al. (1983) (MT protein).

Similar results were obtained when Rop-404 and Rop-Tat were cleaved at Met. The band in Fig. 4, lane 5 (arrow) corresponds to the 7.9-kDa fragment predicted by the nucleotide sequence. The band above the arrow corresponds to the 10.3-kDa fragment obtained from the cleavage of the residual lysozyme in the extract. Unfused Tat protein, obtained by cleavage at Met (aa 53 in Rop-Tat, Fig. 3), is shown in lane 7 (arrow). This fragment was also sequenced through the first 13 aa to confirm the identity of the protein. The structures of Rop-MT and Rop-MT(opp) were confirmed by their cleavage profile after treatment with CNBr.

(g) Fusion protein induction and accumulation

All five fusion constructs produced proteins at consistently high levels if the starter cultures were diluted at least 5000-fold. This was the minimum dilution necessary to achieve high-level plasmid amplification and concomitant protein production. Fig. 5 illustrates the strong correlation between fusion protein accumulation (5A) and plasmid DNA amplification (5B). Plasmid DNA levels reach 20–30 μg/ml of growth medium after 36 h.

The factors that cause some proteins to accumulate in bacteria as insoluble aggregates are poorly understood, but the factor that most of the reported proteins have in common is high cellular concentration (Marston, 1986). To determine if a soluble protein component existed early in induction growth at lower protein concentrations, the samples were separated into soluble and insoluble protein fractions at the 24-h and 48-h time points. As can be seen in Fig. 5C, no soluble (S) fusions are evident in the Rop-Tat or Rop-MT samples at either time point even though cellular levels are lower at the 24-h points. For accurate comparisons the insoluble fractions required carboxymethylation (IAA samples) to prevent precipitation (gel slot of Tat, 48 h insoluble sample) or streaking (24 and 48 h insoluble MT samples) during electrophoresis. The

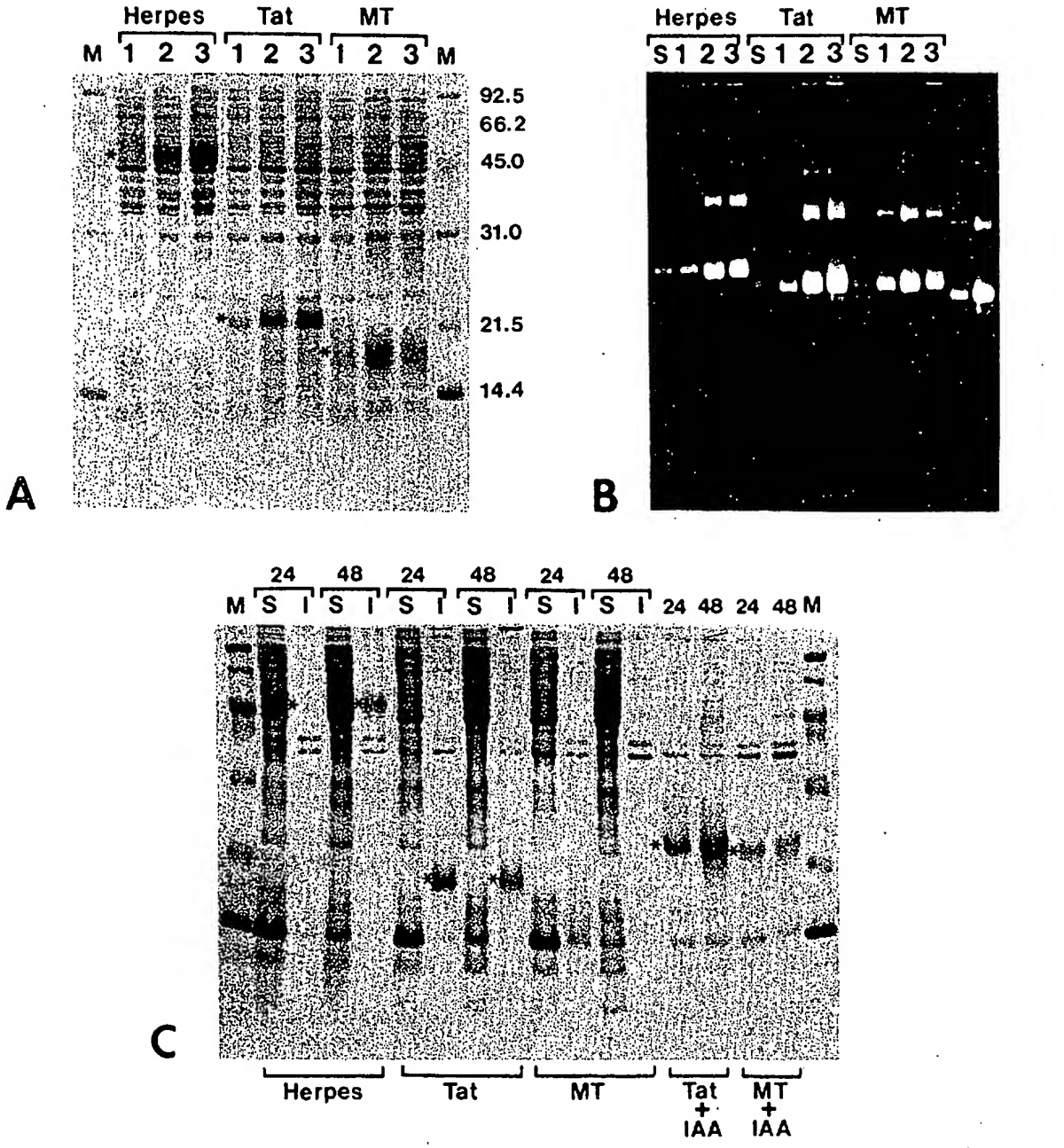

Fig. 5. Analysis of fusion protein induction and accumulation. (Panel A) 0.1% SDS/15.5% polyacrylamide gel of total SDS-soluble protein. Induction growth was initiated by inoculating 10 μl of starter culture (same growth conditions as in Fig. 2) in 50 ml of YT medium (500 μg Ap/ml). Flasks were shaken at 30°C and 1-ml samples were removed at 24 h (lanes 1), 36 h (lanes 2), and 48 h (lanes 3). Cells were pelleted and dissolved in sample loading buffer (Laemmli, 1970). (Panel B) 1% agarose gel of supercoiled plasmid DNA. At the same time points as in Fig. 5A, 1 ml samples were removed and plasmids were extracted (Birnboim and Doly, 1979). Each lane contains plasmid DNA from 5×10^7 cells (approximately 40 μl of culture in lanes marked 2 and 3). Lanes marked S contain DNA extracted from starter cultures. The last two lanes (next to MT samples) contain respectively 0.1 and 1.0 μg of expression plasmid DNA. Gel was stained with ethidium bromide (0.5 μg/ml). (Panel C) 0.1% SDS/15.5% polyacrylamide gel of protein fractionation. At 24 and 48 h, portions of the cultures were removed, and cells were pelleted, washed, lysozyme treated, and sonicated (Kaplan and Greenberg, 1987). After the 100000 × g centrifugation, the soluble (S) supernatant proteins were concentrated by precipitation in 5 vols. of acetone, and dissolved in sample loading buffer. The insoluble pellet (I) was dissolved in sample loading buffer containing 5 M urea. IAA samples are those that were carboxymethylated by treatment with iodoacetic acid after complete reduction in dithiothreitol (Marks et al., 1987). All sample lanes of polyacrylamide gels (stained as described in MATERIALS AND METHODS, section f) contain protein from 2×10^7 cells, or an average volume of 16 μl of culture in the 36 and 48 h time points. Fusion proteins are marked by asterisks. Herpes, HSV protein.

Rop-HSV 38K protein has very little accumulation at 24 h; however, detectable amounts of the protein are present in the insoluble extract. At 48 h 40–50% of this protein remained in the soluble fraction and was the only fusion that behaved in this manner. All the fusions appear to accumulate as insoluble aggregates early in induction growth and do not seem to form simply because of high cellular concentrations.

These experiments illustrate the need to incubate cultures longer than 24 h to achieve maximum levels of accumulation even though the cultures were approaching stationary phase by this time. This requirement is apparent in the pRop-HSV 38K induction, since little fusion protein has accumulated at 24 h. We found that by varying the dilution of the starter culture, each of the five fusions could be fine-tuned to yield maximum cellular fusion accumulation and maximum culture density. We have consistently achieved protein yields of between 100 and 170 μg/ml after 40 h of incubation at 30°C.

(h) Conclusions

The other Rop protein overproducers described in the literature utilize the λ p_L promoter to produce Rop at purified yields of 30–60 mg/100 g of cells (Lacatena et al., 1984; Tomizawa and Som, 1984). Yields of 2–3 μg/ml have been reported using the vector pMAM7 (Muesing et al., 1984; Dooley and Polisky, 1987). These expression systems required several steps to purify the protein. We have produced Rop protein at levels of 150–250 μg/ml of culture (19–31 mg/g of packed cells) with a one-step purification. This system is therefore a powerful method of producing this functionally important protein.

A large number of eukaryotic proteins that accumulate as soluble protein or insoluble aggregates using a variety of bacterial expression systems have been described (Marston, 1986). Several systems combined the features of the thermoinducible runaway plasmid pKN402 (Uhlin et al., 1978) and efficient promoters to produce high-level expression in bacteria (Bittner and Vapnek, 1981; Masui et al., 1983; Remaut et al., 1983). Few of these systems, however, consistently produce proteins as insoluble aggregates as predictably as this high-copy-number *rop* fusion system. In addition, systems utilizing pKN402 sometimes suffer from problems we have often encountered with high temperature runaway-replication: plasmid instability (loss of plasmid or plasmid deletions) and host growth impairment (Uhlin et al., 1978; Remaut et al., 1983). After several inductions with more than ten different constructs, we have not detected plasmid deletions, growth impairment, or premature shutdown of protein synthesis.

Expression systems consisting of fusions with the *trpE* gene (Marston, 1986; Hoffman et al., 1987; Muesing et al., 1987) or *lacZ* gene (Guo et al., 1984; Marston, 1986) usually produce insoluble fusion proteins at levels comparable to the system we have developed. These systems utilize 188 to 320 aa of TrpE and approximately 550 aa of β-galactosidase to produce truncated proteins with yields as high as 200 μg/ml. In contrast, the Rop moiety is quite small, and thus the yields of the sequence of interest are significantly higher, especially in the case of small proteins like MT and Tat.

The expression system we have developed produces insoluble proteins at consistently high yields, on a scale ranging from 5 ml to several liters, without temperature shifts, inducers, or specialized growth media. The cultures do not require high aeration; therefore, fairly large volumes can be cultivated without requiring fermentation equipment. The Rop moiety did not mask or interfere with the antigenic determinants of fused Spec protein (Muesing et al., 1984); therefore, partially purified proteins can be used to produce antibodies in the same manner as TrpE and β-galactosidase fusions. If necessary, the Rop portion of the protein molecule can be removed by chemical methods. Purification of the fragmented proteins is simplified because the cleavage products of Rop are quite small. The system is very stable, and yields of each fusion can be optimized simply by varying the dilution of an overnight culture. It may be especially valuable in the production of small proteins and polypeptides that are frequently degraded in bacteria.

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Antioxidants Inhibit Stimulation of HIV Transcription

FRANK J. T. STAAL, MARIO ROEDERER, PAUL A. RAJU, MICHAEL T. ANDERSON, STEPHEN W. ELA, LEONARD A. HERZENBERG, and LEONORE A. HERZENBERG

ABSTRACT

In studies presented here, we demonstrate that antioxidants regulate NF-κB activation and signal transduction pathways leading to HIV expression. We show (1) that *N*-acetyl-L-cysteine (NAC), an antioxidant and an efficient glutathione (GSH) precursor, inhibits NF-κB activation and HIV expression under conditions in which GSH is depleted and NAC cannot be converted to GSH, (2) that the D-stereoisomer of NAC and a wide variety of chemically unrelated antioxidants also inhibit NF-κB activation and/or transcription directed by the HIV LTR, and (3) that depletion of GSH, the principal intracellular antioxidant, augments HIV production in an acute infection model. Taken together, these findings suggest direct antioxidant action as the mechanism for inhibition of HIV transcription by NAC. They also confirm that GSH, acting in its capacity as an antioxidant, regulates HIV expression and that exogenous antioxidants can potentiate this regulation.

INTRODUCTION

Studies from several laboratories indicate that the signal transduction pathways leading to NF-κB activation and HIV production are redox regulated.[1-7] *N*-Acetylcysteine (NAC), a well-known antioxidant used to replenish intracellular glutathione (GSH) *in vivo* and in cells cultured *in vitro*,[8-10] has been shown to inhibit cytokine-stimulated HIV transcription and replication in a variety of model systems.[1-5] For example, NAC inhibits stimulation of transcription directed by the HIV long terminal repeat (LTR) in reporter gene systems and inhibits cytokine-stimulated HIV production both in acutely infected peripheral blood mononuclear cells (PMBCs)[1] and in chronically infected cell lines.[5] Furthermore, NAC inhibits the activation of the transcription factor Nuclear Factor-κB (NF-κB), which is essential for the stimulated expression of genes controlled by the HIV LTR.[3,6,7] Finally, studies by Baeuerle and colleagues[7] and confirmed in our laboratory (M.T. Anderson, manuscript in preparation), demonstrate that hydrogen peroxide specifically activates NF-κB in certain cell lines and thus clearly implicate oxidants in signal transduction leading to transcriptional activation of the HIV LTR.

Normally, GSH serves as the major scavenger for intracellular oxidants in addition to playing a variety of other roles in cellular metabolism (reviewed in Ref. 11). Because NAC is a GSH precursor, the inhibition of HIV transcription by NAC could be caused by its conversion into GSH, which subsequently acts as a scavenger. Alternatively, NAC could act directly as an antioxidant and not by increasing the availability of GSH. Here we demonstrate that NAC inhibits HIV LTR-directed transcription under conditions in which GSH biosynthesis is inhibited. Moreover, we show that the D-stereoisomer of NAC, which is not a GSH precursor, is as effective as the L-enantiomer. Thus, by two independent assays, we demonstrate that the property of NAC as an antioxidant is responsible for the inhibition of cytokine-induced HIV transcription.

We also show, in accordance with this model of direct reduction (i.e., without conversion into GSH), that a wide variety of antioxidants other than NAC can inhibit transcription directed by the HIV LTR. Furthermore, we show that depletion of GSH augments HIV expression in an acute infection model. These results confirm that antioxidants in general, and GSH in particular, regulate HIV expression.

MATERIALS AND METHODS

Cells. Derivation of the HIV-*lacZ* reporter cell line, 293.27.2, has been described.[1] Human peripheral blood mononuclear cells were obtained by Ficoll-Hypaque density centrifu-

Department of Genetics, Stanford University School of Medicine, Stanford, California 94305.

gation from whole-blood buffy coats purchased from the Stanford Blood Bank. The 293.27.2 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% (v/v) FCS and 5% (v/v) horse serum, L-glutamine, penicillin, and streptomycin.

Stimulation of cells. 293.27.2 cells were plated in individual wells of 96-well Costor (Cambridge, MA) plates at a density of 5000 cells/well in 0.2 ml of medium. The next day, phorbol myristate acetate (PMA; Sigma, St. Louis, MO), tumor necrosis factor α (human rTNF-α; Cetus, Emeryville, CA), L-NAC (Aldrich, Milwaukee, WI), D-NAC (kindly provided by Dr. A. Tunek, Astra Draco, Lund, Sweden), or *N*-acetylserine (NAS; Sigma) were added to the desired concentrations from stock solutions. The enantiomeric purity of L- and D-NAC was confirmed by measurement of optical rotation: $[\alpha]^{25}$ for D-NAC = -4.6 ± 0.3 and $[\alpha]^{25}$ for L-NAC = $+ 4.4 \pm 0.3$. Stimulations were for 6 or 8 hr at 37°C,5% CO_2. In experiments with buthionine sulfoximine (BSO; Sigma), cells were pretreated with BSO (100 μM) for 2 days with daily refeeding (including BSO), and received BSO at the start of the stimulations as well. Experiments with the panel of antioxidants were performed as with NAC. Glutathione, glutathione disulfide (GSSG), L-cysteine, L-cystine, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), sodium diethyldithiocarbamate (DDTC), nordihydroguaiaretic acid (NDGA), vitamin E succinate, and sodium ascorbate were all from Sigma. L-2-Oxothiazolidine-4-carboxylic acid (OTC) was from Clintec Nutrition. All stocks were neutralized to pH 7.0 prior to use.

Measurement of β-galactosidase activity. The 4-methylumbelliferyl β-D-galactoside (MUG) assay of β-galactosidase was carried out as described.[12]

Nuclear protein extracts and electrophoretic mobility shift assays. Nuclear protein extracts were made essentially as described,[6,13] with some modifications to optimize conditions for our cells. Cells (10^7) were harvested after stimulation for 2 hr, centrifuged (10 min, 1200 rpm, 4°C), and washed in 1 ml of ice-cold Tris-buffered saline. All subsequent steps were done in the cold room and on ice. Cells were pelleted and washed once in 0.4 ml of buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 3 mM dithiothreitol (DTT), 0.1 mM EDTA (ethylenediaminetetraacetic acid) 0.1 mM phenylmethylsulfonyl fluoride (PMSF)], supplemented with protease inhibitors and incubated on ice for 17 min. Then 25 μl of a 10% Nonidet P-40 (NP-40) solution was added and cells were vigorously mixed and centrifuged. Pelleted nuclei were resuspended in buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 3 mM DTT, 0.1 mM PMSF, 10% (v/v) glycerol] and gently mixed for 30 min, after which samples were centrifuged for 10 min at 14,000 rpm to obtain clear supernatants containing the nuclear proteins. Protein samples were stored at −70°C. Electrophoretic mobility shift assays (EMSAs) were done as described[14] with 1.0 μg of nuclear extract and labelled κB probes under experimentally determined optimal binding conditions (final salt concentration, 70 mM; DTT concentration, 5 mM; 0.2 μg dIdC). Samples were run on 4.5% acrylamide gels and bands were visualized by autoradiography and quantitated by direct counting of radioactivity with a radioanalytic imaging system (Ambis Systems, Inc).

Monitoring of HIV infection. Peripheral blood mononuclear cells were maintained in complete RPMI-1640 medium [supplemented with 20% heat-inactivated fetal calf serum (FCS), glutamine, and antibiotics]. The PMBCs (3×10^6/ml) were stimulated for 3 days with 2 mg of phytohemagglutinin (PHA)/ml and 5% purified human interleukin 2 (IL-2; Pharmacia, Piscataway, NJ) in complete medium. Cells were resuspended at 1×10^6/ml in complete RPMI with IL-2, and with or without 100 μM BSO. After 3 days cells were pelleted, and infected by incubation for 45 min at 37°C with the supernatant from TNF-α-stimulated ACH-2 cells and 3 μg of Polybrene/ml. Cells were then washed twice with RPMI and resuspended at 1×10^6/ml in complete RPMI with IL-2 and supplemented with TNF-α (10 ng/ml), PMA (20 ng/ml), BSO (100 μM), and/or the indicated concentrations of NAC or OTC. After 48 hr, cells were pelleted, counted, and resuspended at 1×10^6/ml in medium supplemented identically for continued culture. Twenty-four hours later, aliquots of the medium were collected for p24 measurements by enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories, Abbott Park, IL).

RESULTS

NAC inhibits HIV LTR-directed transcription, whereas NAS does not

Using a reporter cell system in which the HIV LTR directs expression of β-galactosidase, we have demonstrated previously that TNF-α- and/or PMA-induced HIV transcription is effectively inhibited by NAC (see Ref. 1; similar data from different experiments are presented in Fig. 1A and Fig. 3).

Because rather high (10–30 mM) concentrations of either D- or L-NAC are required to inhibit expression of the HIV LTR-I *lacZ* construct in the 293.27.2 cell line, we used *N*-acetylserine (NAS) to control for nonspecific inhibitory effects, for instance, effects of osmolarity. *N*-Acetylserine differs from NAC only in that it contains a hydroxyl group instead of a sulfhydryl group. As shown in Fig. 1A&B, NAC inhibits the TNFα- and/or PMA-induced activation of the HIV LTR, whereas the same concentrations of NAS show no inhibition. In addition, 30 mM OTC, a non-reducing cysteine precursor, also had no inhibitory effect. Thus, the inhibition is specifically brought about by the reducing thiol group of NAC rather than by nonspecific effects due to the presence of a high concentration of reagent.

NAC inhibits HIV LTR-directed transcription when GSH synthesis is blocked

N-Acetylcysteine is a GSH precursor[10,15] and could potentially inhibit HIV LTR-directed transcription by raising (or by preventing a decrease of) intracellular GSH. However, NAC is an antioxidant by itself and it could act as a direct reductant and free radical scavenger.[8] To try to distinguish between these possibilities, we carried out two different types of experiments. In the first experimental approach we utilized BSO, an inhibitor of γ-glutamylcysteine synthetase, one of the key enzymes in GSH biosynthesis.[16] Buthionine sulfoximine is frequently used to lower GSH levels in cells, but here we used it to ensure that NAC (or cysteine after deacetylation) will not be converted into GSH. The BSO-treated cells (100 μM, 48 hr) also have lower

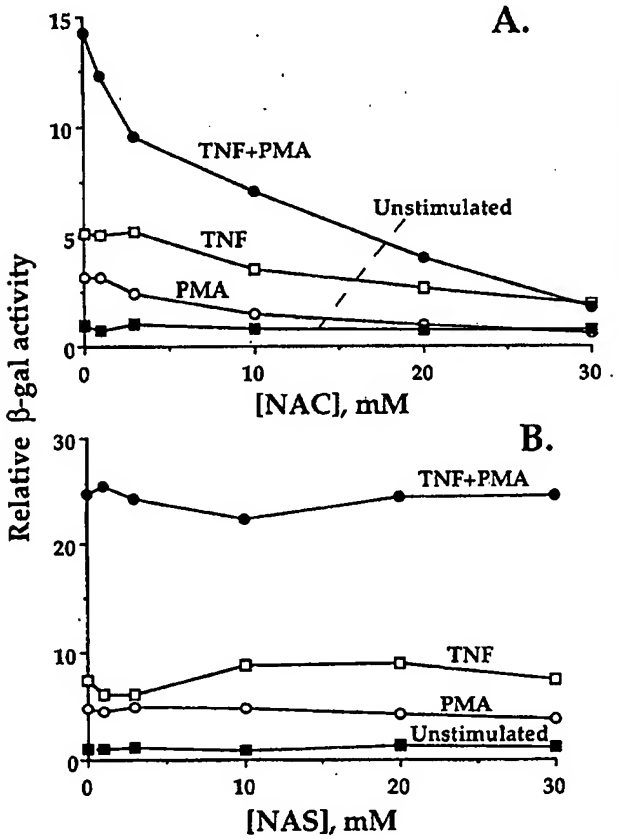

FIG. 1. NAC inhibits HIV LTR-directed transcription, but NAS does not. 293.27.2 cells, containing a construct in which the HIV LTR directs β-galactosidase expression, were stimulated as described in Materials and Methods. All stimulations were for 6 hr. Titration curves for NAC (A) and NAS (B) were established, showing that NAS does not inhibit HIV LTR-directed transcription in this reporter gene system, whereas NAC shows a concentration-dependent inhibition.

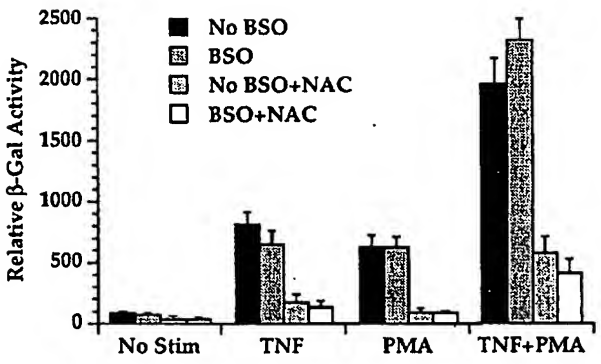

FIG. 2. NAC inhibits HIV LTR-directed transcription as measured by β-galactosidase in the presence of BSO. BSO inhibits biosynthesis of glutathione and lowers GSH levels in cells. Cells were pretreated with BSO (100 μM) for 2 days (see Materials and Methods). When 293.27.2 cells are cultured under conditions in which GSH synthesis is impossible, NAC still is inhibitory for HIV transcription. Error bars indicate the standard deviation of four replicates for each condition.

GSH levels (about 10% of normal, data not shown), showing that BSO is indeed active under these conditions. As shown in Fig. 2, NAC inhibits stimulation of the reporter construct in both BSO-treated cells and the parallel cultures that are untreated. The degree of inhibition by NAC is essentially the same with or without BSO. Thus, when *de novo* GSH biosynthesis is inhibited, NAC still inhibits stimulation of HIV LTR-directed transcription, suggesting that NAC works as a direct antioxidant under these conditions and not through conversion into GSH.

D-NAC inhibits HIV transcription to the same degree as L-NAC

In the second type of experiment we used the D-stereoisomer of NAC (D-NAC, *N*-acetyl-D-cysteine). This stereoisomer, which cannot be converted into GSH because the enzymes that are involved in GSH biosynthesis use only L-amino acids, has been used by others to address the same question (direct antioxidant or GSH precursor) in different experiments, for example, in studies using NAC as an antidote against acetaminophen-induced liver toxicity.[17] In those studies, the D-stereoisomer was ineffective, showing that conversion of L-NAC to GSH was required for protection. In contrast, we find that D-NAC and L-NAC both inhibit HIV LTR-directed transcription, and essentially to the same degree (Fig. 3). In fact, D-NAC is slightly more inhibitory, possibly because enzymes that metabolize L-NAC attack the D-form much less efficiently,[18] and thus higher levels of D-NAC are maintained. In conclusion, these experiments show in two independent ways that NAC can act as a direct antioxidant to inhibit HIV LTR-directed transcription in this reporter gene system.

Other antioxidants also inhibit HIV LTR-directed transcription

Because NAC apparently works as a direct antioxidant in the inhibition of HIV transcription, we determined whether other antioxidants could also inhibit HIV LTR-directed transcription in our β-galactosidase reporter gene system. Table 1 shows that L-cysteine and glutathione (GSH), but not their oxidized disulfides L-cystine and GSSG, inhibit HIV transcription. Other antioxidants that do not contain a sulfhydryl group, such as BHA, NDGA, vitamin E succinate, and reduced vitamin C (ascorbate), also inhibit the cytokine-stimulated HIV transcription. Inhibition of HIV transcription by ascorbate has also been reported by Harakeh and Jariwalla,[19] who have further demonstrated synergism between NAC and ascorbate *in vitro*. The BHA-related compound BHT did not inhibit HIV transcription. Both BHA and ascorbate are cytotoxic at concentrations 5- to 10-fold higher than their IC_{50} (50% inhibitory concentration). The thiol compounds were not found to be cytotoxic, but are cytostatic at concentrations higher than the IC_{50} for both TNF-α and PMA.

Oxothiazolidine-4-carboxylic acid is not an antioxidant, but is a GSH precursor, because it is converted intracellularly to L-cysteine through the action of 5-oxoprolinase.[20] Cysteine is subsequently used to synthesize GSH. This compound did not inhibit HIV transcription in the reporter gene model (Table 1);

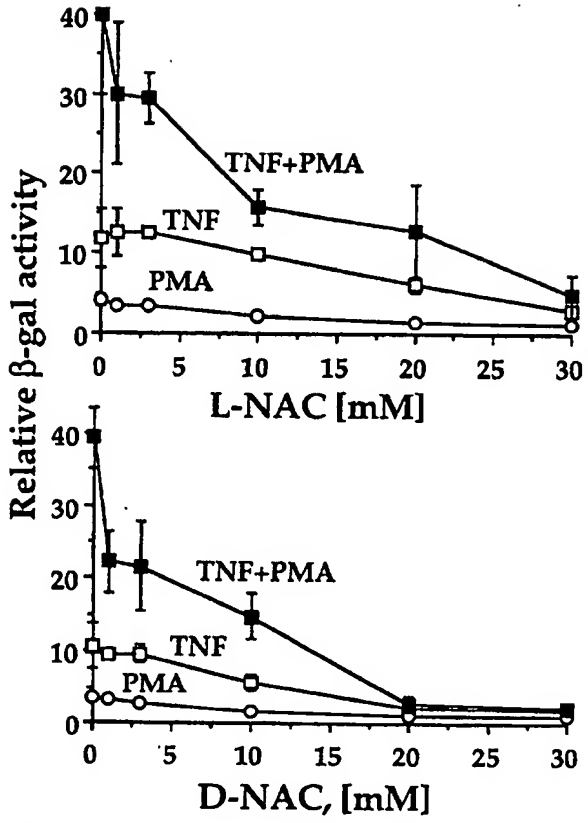

FIG. 3. L-NAC (*top*) and D-NAC (*bottom*) both inhibit HIV LTR-directed transcription measured as β-galactosidase activity. The D-stereoisomer of NAC cannot be used for GSH biosynthesis, but still inhibits HIV transcription to the same degree as the L-stereoisomer. For experimental details see text. Error bars indicate the standard deviation of five replicates for each point.

apparently the 5-oxoprolinase activity is too low to provide enough cysteine (within the time span of the assay), which then could act as an antioxidant.

We also tested DDTC (Ditiocarb sodium, Imuthiol), a non-thiol-containing antioxidant with strong metal-chelating properties that has been used in clinical trials for HIV infection.[21] We observed strong inhibition of HIV transcription at low concentrations (IC_{50}, ~10 μM); however, even slightly higher concentrations were toxic to the cells. In conclusion, we show that a wide variety of antioxidants (sulfhydryl-containing compounds, nonsulfur antioxidants, and metal chelators) are capable of inhibiting HIV transcription *in vitro*. This supports the assertion that, in this model system, GSH synthesis is not required for inhibition by NAC.

Both L-NAC and D-NAC inhibit NF-κB activation

We and others have reported previously that L-NAC can inhibit the cytokine-induced activation of the cellular transcription factor NF-κB.[3,6,7] This transcription factor is used by HIV to markedly up-regulate transcription of its genes and to enhance viral replication.[22] Because D-NAC inhibits HIV transcription in a β-galactosidase reporter gene system (as described above), we tested if D-NAC can also inhibit activation of the NF-κB transcription factor. Nuclear protein extracts were prepared from 293.27.2 cells stimulated with TNF-α, PMA, or both in the presence or absence of 30 mM L- or D-NAC and gel retardation assays were done. Both enantiomers effectively inhibited activation of NF-κB, and both are more effective in inhibiting PMA-induced activation than TNF-induced activation (Fig. 4). The inhibition of NF-κB activation by both stereoisomers is in agreement with data from the HIV LTR–reporter gene experiments and provides a partial explanation in terms of a molecular mechanism for the observed inhibition of HIV transcription in that system.

TABLE 1. INHIBITION OF HIV LONG TERMINAL REPEAT-DIRECTED TRANSCRIPTION BY ANTIOXIDANTS[a]

Compound	*Toxic level*[b]	*Approximate IC_{50} for stimulation*		
		TNF-α	*PMA*	*TNF-α + PMA*
NAC (L- and D-)	>50 mM	15 mM	7 mM	15 mM
L-Cysteine	20 mM	15 mM	7 mM	15 mM
L-Cystine10 mM	10 mM		No inhibition	
GSH	>50 mM	20 mM	20 mM	10 mM
GSSG	10 mM	*	*	*
DDTC	0.02 mM	0.10 mM	0.010 mM	0.010 mM
NDGA	0.30 mM	0.1 mM	0.005 mM	0.1 mM
Vitamin E succinate	0.25 mM	0.1 mM	0.025 mM	0.1 mM
Ascorbate (vitamin C)	6 mM	5 mM	3 mM	3 mM
BHA	1 mM	0.1 mM	0.050 mM	0.05 mM
BHT	1 mM	*	*	*
OTC	>30 mM	*	*	*

[a] 293.27.2 cells were stimulated with TNF-α (10 ng/ml), PMA (20 ng/ml), or both as described in Methods. A range of concentrations of the above compounds was tested and the concentration that gave 50% inhibition (IC_{50}) of HIV transcription was determined from the titration curves. Compounds that showed no inhibition were tested for concentrations up to 30 mM (GSSG, L-cystine, OTC) or until cytotoxic levels were reached (millimolar range for BHT).

[b] Toxic levels were defined as viability < 90% after a 24 hr incubation period with the compound.

*No inhibition

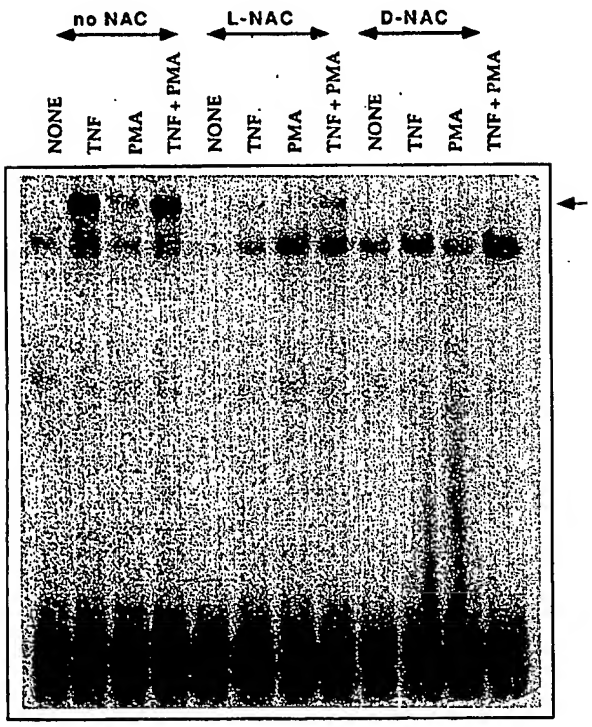

FIG. 4. D-NAC inhibits NF-κB activation to the same degree as L-NAC. Nuclear protein extracts were prepared from 293.27.2 cells after stimulation for 2 hr in the presence or absence of D-NAC or L-NAC. Retardation assays were done as described in Materials and Methods. The arrow indicates the inducible NF-κB band; free probe runs at the bottom of the gel.

Acute, in vitro HIV infection is inhibited by both D-NAC and L-NAC

Results described above establish the efficacy of D-NAC in model systems for HIV infection. We have demonstrated earlier that L-NAC inhibits HIV infection of normal (HIV^-) PBMCs at low (<1 mM) concentrations. As shown in Table 2, D-NAC is as effective as L-NAC in inhibiting viral replication after acute infection *in vitro*. In fact, a 1 mM concentration of either stereoisomer inhibits, virtually completely, viral production in this experiment. Data from other experiments with L-NAC are given in Table 3, showing that 1 mM L-NAC inhibits viral replication by ~90%. In contrast, the GSH prodrug OTC, even at a 10 mM concentration, only partially inhibits HIV replication (Table 3), again demonstrating the importance of antioxidant properties for the compounds that efficiently inhibit HIV replication and transcription.

TABLE 2. *N*-ACETYL-D-CYSTEINE AND *N*-ACETYL-L-CYSTEINE INHIBIT HIV REPLICATION IN VITRO[a]

Stimulation condition	*No antioxidant p24 production*	*L-NAC*[b]	*D-NAC*[b]
No stimulation	40	<0.1	<0.1
TNF-α	60	<0.1	<0.1
TNF-α + PMA	107	<0.1	<0.1
BSO	57	<0.1	<0.1
TNF-α + BSO	117	<0.1	<0.1
TNF-α + PMA + BSO	131	<0.1	<0.1

[a] Normal (HIV^-) PBMCs were infected *in vitro* with HIV as described in Materials and Methods. Inhibition measured as p24 production (in nanograms per milliliter).
[b] 1mM of both D-NAC and L-NAC was added.

TABLE 3. INHIBITION OF STIMULATED VIRAL REPLICATION IN PERIPHERAL BLOOD MONONUCLEAR CELLS BY *N*-ACETYLCYSTEINE AND L-2-OXOTHIAZOLIDINE-4-CARBOXYLIC ACID[a]

Treatment	*Concentration (mM)*	*HIV production (% of untreated)* *Expt. 1*	*Expt. 2*	*Expt. 3*
NAC	1	15	13	8
OTC	1	74	88	nd
	3	62	nd	nd
	10	46	75	43
	30	5	nd	nd

[a] Peripheral blood mononuclear cells were prepared and infected as in Materials and Methods, and stimulated with TNF (10 ng/ml) and PMA (20 ng/ml) with or without the indicated concentrations of NAC or OTC. The amount of p24 produced in the presence of NAC or OTC is shown as a percentage of that produced without either drug. nd, not done.

GSH depletion enhances HIV production

Because HIV-infected individuals have low levels of intracellular GSH in T cells,[23] we tested the effect of lowering GSH levels in normal PBMCs on acute viral infection. When the cells are cultured in the presence of BSO (100 μM), intracellular GSH levels are lowered to about 60% of normal under these conditions (data not shown). Under all conditions, cell viability and growth were identical. Data in Table 2 show that the viral production is enhanced in cells with lower GSH levels (as compared to cells with normal GSH levels). This enhancement is observed under stimulated and unstimulated conditions. This suggests that the low GSH levels seen *in vivo* (in patients) may contribute to a higher rate of HIV replication.

DISCUSSION

Studies presented here demonstrate that the cytokine-stimulated signal transduction pathway leading to NF-κB activation and HIV transcription is redox regulated. In previous studies, we have shown that the transduction of these signals is inhibited by NAC, an antioxidant that is efficiently converted to GSH and used medically to replenish GSH. The key regulatory role suggested for GSH by these findings is confirmed here by studies that show that depletion of GSH increases HIV expression in PBMCs. In addition, studies presented here demonstrate that NAC works as a direct antioxidant to inhibit HIV transcription and replication. A number of other antioxidants with different chemical structures also inhibit stimulated HIV transcription (in a reporter gene system).

The most likely way that L-NAC could be useful for treatment *in vivo* is through restoration of GSH levels. HIV-infected people have lower plasma and intracellular GSH (reviewed in Ref. 24), and NAC may restore this deficiency. High GSH levels may keep the virus in a silent state (by blocking inappropriate NF-κB activation) and prolong clinical latency. The potential use of L-NAC as a therapeutic agent for HIV infection has been reviewed elsewhere.[24–27] Because D-NAC is not a GSH precursor, we do not regard it as a potential therapeutic agent for HIV disease.

N-Acetylcysteine (and other antioxidants) could have an intracellular or extracellular mode of action. Because D-NAC is deacetylated at a much slower rate than L-NAC,[18] the intracellular action of D-NAC is likely through D-NAC itself, whereas L-NAC probably partially acts as L-cysteine. (Note that L-NAC, D-NAC, and L-cysteine have identical IC_{50} values; see Table 1). These findings confirm and extend the observations from Droge's laboratory that GSH may not be the sole low molecular weight thiol that can modulate HIV expression.[3] We cannot exclude the possibility that L-NAC and D-NAC act extracellularly to facilitate equally the uptake for cystine from the medium into the cells. Precedence for such a mechanism comes from studies by Issels *et al.*,[28] who examined the uptake of [^{35}S]cystine and GSH synthesis in CHO cells. On adding either cysteamine or L-NAC they found increases in radioactivity in the cells and increases in GSH synthesis, supporting the conclusion that NAC and cysteamine acted as delivery systems for cystine from the medium outside the cells into the cells. However, because L-NAC is readily taken up by cells and converted into cysteine,[29] an intracellular mechanism seems more likely, especially because other chemically distinct antioxidants that will not facilitate cystine uptake also inhibit HIV transcription.

Taken together with earlier data from Baeuerle's group that hydrogen peroxide can directly activate NF-κB and that NAC can block NF-κB induction by a wide variety of agents,[7] there is a substantial body of evidence implicating a redox-sensitive step in the signal transduction leading to NF-κB activation and HIV transcription. The molecular nature of such step(s) is as yet unknown, although several can now be ruled out. Schreck et al.[30] reported that the antioxidant pyrrolidine dithiocarbamate (PDTC) strongly inhibits NF-κB activation and HIV transcription but does not interfere with nuclear uptake of NF-κB or with the release of Inhibitor of NF-κB (IκB). In addition, it has been shown that NAC does not interfere with *in vitro* binding of NF-κB to its DNA site.[7] These findings locate the redoxsensitive step within the actual signal transduction pathway leading to release of IκB from NF-κB. Consistent with this hypothesis, preliminary data indicate that there may be a redox-regulated tyrosine phosphorylation step in the transduction of certain signals leading to NF-κB induction (Staal *et al.*, manuscript in preparation).

These findings might be viewed as inconsistent with evidence from three reports demonstrating redox effects on NF-κB binding to DNA *in vitro* (as opposed to regulation of NF-κB activation in intact cells).[31–33] In these reports, oxidants decrease *in vitro* binding to the κB site and reducing agents increase this binding. However, we have shown the opposite for *induction* of NF-κB in intact cells, that is, oxidants stimulate NF-κB activation and reducing agents block this stimulation. This difference can be understood by recognizing that the mechanisms that increase NF-κB binding activity *in vitro* are probably unrelated to the mechanisms that increase the amount of activated NF-κB in the cell. Alternatively, the *in vitro*-binding studies may have relevance to the actual situation within the cell, if one assumes that different redox environments exist in the cytoplasm and the nucleus. Data from Orrenius's group, showing a three-fold higher GSH concentration in the nucleus as compared to the cytoplasm,[34] give some support for this notion.

Transcription factors other than NF-κB can be regulated by redox mechanisms as well. Curran's group has described that the DNA binding of Fos and Jun is redox regulated.[35,36] Reduction of a conserved cysteine residue in the DNA-binding domain of Fos and Jun is required for AP-1 binding. A nuclear redox factor, named Ref-1, has been identified[37] and cloned[38] and is probably responsible for reduction of this cysteine residue. Intriguingly, Ref-1 can also stimulate the DNA-binding activity of NF-κB, as well as other transcription factors (Myb, CREB, ATF-1, ATF-2).[38]

Oxidative stress [e.g., hydrogen peroxide and ultraviolet (UV) irradiation], induces activation of c-*fos* and c-*jun* in intact cells.[39,40] Thus redox regulation of AP-1 is similar to redox regulation of NF-κB, in that oxidation can induce or activate the transcription factor, whereas reduction seems to be required for DNA binding, at least *in vitro*. This again illustrates the complexities of redox regulation, especially in intact cells in which different signal transduction pathways, redox control systems, and cell compartments all contribute to the final readout. In addition to NF-κB and AP-1, the DNA-binding activity of certain other transcription factors, including OxyR,[40] TFIIIC,[41] and some steroid receptors,[42,43] is redox regulated. Thus redox mechanisms may provide a means for control of signal transduction pathways and posttranslational regulation of transcription factor function.

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Address reprint requests to:
Leonore A. Herzenberg
Beckman Center B007
Department of Genetics
Stanford University School of Medicine
Stanford, California 94305

Synthesis of Lignans. I. Nordihydroguaiaretic Acid[1]

CLARK W. PERRY,* MALDA V. KALNINS, AND KENNETH H. DEITCHER

Chemical Research Department, Hoffmann-La Roche Inc., Nutley, N. J. 07110

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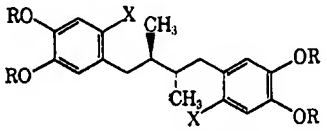
The novel alkylation of the sodium enolate of propioveratrone (4) with α-bromopropioveratrone (5) in liquid ammonia gave the racemic diketone 9. A mechanism for the stereoselectivity of this alkylation is proposed and the structural requirements of the reaction are discussed. Cyclodehydration of 9 to the furan 8 followed by hydrogenation *via* the all-cis tetrahydrofuran 25 afforded nordihydroguaiaretic acid (NDGA) tetramethyl ether (2). A pronounced solvent effect on the hydrogenation was observed. Demethylation of 2 with concentrated hydrobromic acid afforded NDGA (1) in good yield and high purity. An alternate route to 2 *via* the alcohol 28 is also described.

Nordihydroguaiaretic acid (1), more commonly known as NDGA, is a phenolic lignan found in the resinous exudates of many plants, especially *Larrea divaricata*, the creosote bush of the southwestern United States.[2] Its structure was established by synthesis,[3] and there was strong chemical evidence[4] that the naturally occurring optically inactive NDGA was the meso rather than the racemic form.

Several syntheses of NDGA have appeared in the literature[3-8] and in patents,[6,9] but all involve low-yield reactions, lengthy reaction sequences, or expensive starting materials. The creosote bush has remained the only commercial source of NDGA, which has been used as an antioxidant in foods.

This report presents a practical synthetic route to NDGA utilizing a novel and highly stereoselective alkylation reaction to form the lignan carbon skeleton.

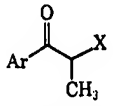
In order to obtain an unequivocal confirmation of the configuration[4] of natural NDGA, the tetramethyl ether 2,[5] prepared from commercial NDGA,[10] was brominated, affording the dibromo derivative 3.[5] A



1, R = X = H (NDGA)
2, R = CH_3; X = H
3, R = CH_3; X = Br

single-crystal X-ray analysis[11] confirmed that NDGA (1) has the meso configuration.

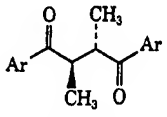
Acylation of veratrole with propionyl chloride using chloroform as solvent gave a higher yield (93%) of purer product, 3,4-dimethoxypropiophenone (4), than previously reported methods.[12,13] Bromination of 4 in refluxing chloroform then gave α-bromo-3,4-dimethoxypropiophenone (5), again in considerably better yield (95%) than by the methods previously described.[13]



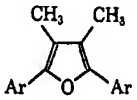
Ar = 3,4-dimethoxyphenyl

4, X = H
5, X = Br
6, X = Cl

Heating 5 with copper powder in refluxing xylene has been reported[14] to give 2,3-diveratroylbutane (7) in 28% yield, but, using a variety of copper powders and solvents, we could obtain the rather insoluble compound 7 in no more than 7% yield. Column chromatography of the mother liquors led to crystalline mixtures containing a second diketone (9) contaminated with 7. Dehydration of either 7 or 9 afforded the furan 8 in high yields. From the data to be presented, it is clear that 7 and 9 are the expected meso and racemic diketones, respectively.



7 (meso)



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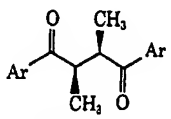
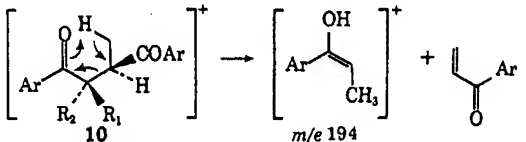


9 (racemic)

Support for the assignments of relative configuration to 7 and 9 came from their mass spectra,[15] which showed that a fragment ion at *m/e* 194 was much stronger in the spectrum of 7 than in that of 9. This ion presumably arises by a rearrangement fragmentation,[16] the transition state (10) for which is sterically more favorable when R_1 = H and R_2 = CH_3 (7) than when R_1 = CH_3 and R_2 = H (9).



10

m/e 194

7, R_1 = H; R_2 = CH_3
9, R_1 = CH_3; R_2 = H

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(15) Dr. F. Vane, Hoffmann-La Roche Inc., unpublished results.
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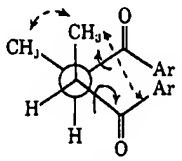
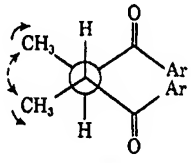
Examination of the infrared spectra of these diastereomeric diketones showed some striking differences. The carbonyl absorption frequencies in the infrared spectra of the following compounds were measured in dilute carbon tetrachloride solution: propiophenone (1692 cm^{-1}), *p*-methoxypropiophenone (1685 cm^{-1}), 3,4-dimethoxypropiophenone (4, 1681 cm^{-1}), the racemic diketone (9, 1669 cm^{-1}), and the meso diketone (7, 1662 cm^{-1}). The lowering of the frequency by 11 cm^{-1} from propiophenone to 4 is clearly the result of electron enrichment of the carbonyl group of 4 by the strongly electron-donating methoxyl substituents, but the further lowering of the frequency by 12 cm^{-1} in 9 must be due to strong interaction (and therefore close proximity) between the two aroyl groups of 9. Even stronger aroyl–aroyl interaction must exist in the meso diketone 7. Such aroyl–aroyl interactions can be understood best in terms of the conformations of the diketones shown below (7a and 9a), in which



7a



9a

the planar aroyl groups lie in approximately parallel planes and in close proximity.

Inspection of Dreiding models of these molecules also suggests that, in the meso diketone conformer 7a, one of the methyl groups interacts sterically not only with the other methyl group, but also strongly with the ortho hydrogen atom on the nearer aryl group, as indicated by the dotted arrows. Both of these interactions are relieved best by slight rotations of the C–aroyl bonds, as indicated by the solid arrows. Such a conformational adjustment is done at the expense of forcing the aroyl groups into closer proximity, which is compatible with the infrared data. On the other hand, the methyl–methyl interaction in the racemic diketone conformer 9a, shown by the dotted arrow, is easily relieved by slight rotation about the central carbon–carbon bond, and this motion tends to decrease the aroyl–aroyl overlap.[17]

The observed differences in the pmr spectra of these diketones are also understood in terms of such conformations. On the average, the methyl groups of the meso diketone conformer 7a are closer to, and therefore more shielded by, the aroyl groups than are the methyl groups of the racemic diketone conformer 9a. Accordingly, the signals for the methyl protons are found at higher field for 7a than for 9a. Base-catalyzed equilibration of either diketone led to mixtures of 7 and 9, their ratio depending on the solvent as shown in Table I.

A better method of preparing the meso diketone 7 was sought in the reaction of the sodium enolate of 4 with the bromo ketone 5. However, in DMSO the reaction gave the racemic diketone 9 in low yield. When the alkylation reaction was carried out in liquid ammonia at −33°, the pure racemic diketone 9 was obtained in 90% yield.

(17) No attempt is made to assess the absolute magnitude of aroyl–aroyl (or aryl–aryl) overlap in these compounds, but only the relative overlap as it is affected by these simple conformational adjustments from the ideal completely staggered conformations drawn in the diagrams above.

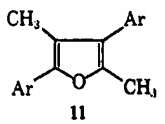
TABLE I

Starting material	Solvent	Base	Time, hr	Temp, °C	Ratio, 9:7[a]
7	C_2H_5OH–CH_3OH–C_6H_6 (4:4:1)	$NaOCH_3$	1	Reflux	1.9
9	C_2H_5OH–CH_3OH–C_6H_6 (4:4:1)	$NaOCH_3$	1	Reflux	2.2
7	DMSO	KO-*t*-Bu	1	22	3.6
9	DMSO	KO-*t*-Bu	1	22	3.9
7	C_6H_6	KO-*t*-Bu	1	40	2.8
9	C_6H_6	KO-*t*-Bu	1	40	2.4

[a] Differences in ratios in the same solvent systems are within experimental error.

As expected from the preceding analysis of the steric interactions in the two diastereomeric diketones, the racemic form 9 was found in each case to be slightly favored over the meso form 7. However, the observed difference in stability does not account for the highly stereoselective alkylation of 4 by 5 to give the racemic diketone almost exclusively.

The novel and highly stereoselective alkylation of 4 by 5 in liquid ammonia seems to have rather specific structural requirements. When the α-chloro ketone 6 was used in place of 5, the major product isolated (*ca.* 15% yield) was the furan 11, perhaps arising *via* cyclization of an intermediate such as 12 resulting from a Darzens-type condensation. When the substituted acetophenones 13 and 14 were condensed under the same conditions, the reaction took yet another course, affording 15 and 16 in low yields along with recovered 13, but none of the expected diketone 17.



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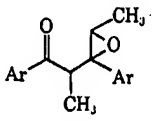
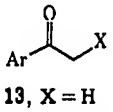
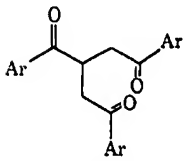


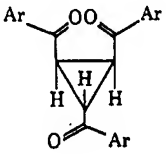
12



13, X = H
14, X = Br



15



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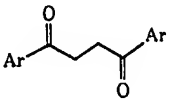
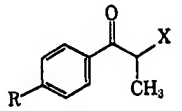


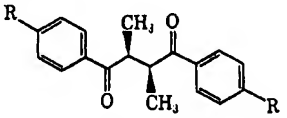
17

Assignment of structures 15 and 16 was based mainly on elemental analysis, and pmr and mass spectra. The presence of three, rather than two, pmr signals for the methoxy groups of 16 supports the trans configuration assigned.

On the other hand, the condensation proceeded normally with the pairs of propiophenone derivatives 18–19 and 20–21, giving in each case the corresponding racemic diketones, 22 and 23, in good yields. The meso diketones were not detected.

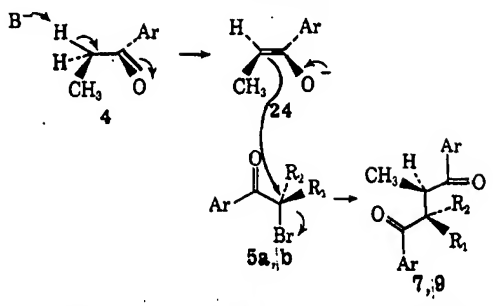


18, R = X = H
19, R = H; X = Br
20, R = OCH_3; X = H
21, R = OCH_3; X = Br



22, R = H
23, R = OCH_3

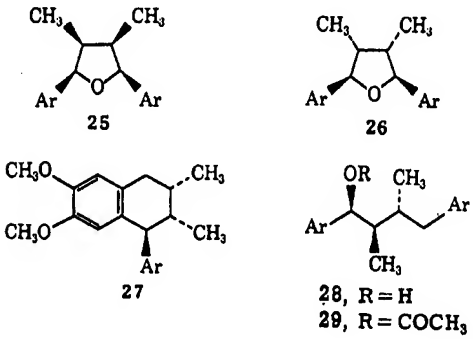
CHART I



5a, $R_1 = H$; $R_2 = CH_3$ 7, $R_1 = H$; $R_2 = CH_3$ (meso)
5b, $R_1 = CH_3$; $R_2 = H$ 9, $R_1 = CH_3$; $R_2 = H$ (racemic)

The stereoselective formation of the racemic diketones in these reactions may be rationalized by the following proposed mechanism. Formation of the enolate from 4 should give predominantly the trans enolate 24. Reaction of 24 with either enantiomer 5a or 5b from either above or below 24 is possible, but it would be sterically more favorable when the aroyl group of 5 is farthest from the aroyl group of 24, and the methyl group of 5 is toward the oxygen of 24 rather than toward the aryl group. Thus, in the example illustrated in Chart I, reaction would be more likely to occur with 5b than with 5a, and displacement of bromide by the enolate with Walden inversion would lead to one enantiomer of the racemic diketone 9. In like manner, and with equal probability, approach of 5 on the top side of 24 would preferentially form the other enantiomer of 9, with the net result that the product is predominantly the racemic form.

Catalytic hydrogenation of the furan 8 was reported[8] to give either NDGA tetramethyl ether (2) or the all-cis tetrahydrofuran 25 depending on conditions, but no yield was given for the first case, and 70% was reported for the second. During extensive efforts[18] to hydrogenate 8 directly to 2, an improved procedure for the preparation of 25 in 89% yield was developed, but none of the catalysts or conditions studied gave 2 in significant yields.



25 26 27 28, R = H 29, R = $COCH_3$

Galgravin (26), prepared by acid-catalyzed isomerization of 25, is reported to be more easily hydrogenated to 2 with palladium oxide than is the furan 8.[8] A series of similar hydrogenation experiments, summarized in Table II, was conducted on 8 and 25, and disclosed a remarkable solvent effect on the ratio of the two products formed, 2 and racemic isogalbulin (27).[19] The formation of 27 may be the result of desorption of the intermediate alcohol 28 from the catalyst followed by its cyclodehydration in solution. Relatively nonpolar solvents such as THF may cause less desorption of 28; so relatively more 2 is formed.

TABLE II

Substrate	Catalyst	Solvent	Temp, °C	Time, hr	Products, % 2	27
8[c]	PdO	AcOH	25	71	28[a]	58[a]
25[c]	PdO	AcOH	25	2	25[a]	75[a]
25	PdO	MeOH	25	20	5[a]	95[a]
25	PdO	EtOAc	25	23	74[a] 56[b]	26[a]
25	PdO	THF	25	45	79[a] 76[b]	21[a]
25	PdO	C_6H_6	25	24	No reaction	
8	PdO	THF	75 (1500 psig)	0.5	81[a]	18[a]

[a] Determined by gas chromatography. [b] Isolated crystalline. [c] Conditions similar to those reported by Blears and Haworth.[8]

As seen from the last entry in Table II, the use of tetrahydrofuran (THF) as a solvent and palladium oxide as a catalyst, along with elevated temperature and pressure, permitted the direct conversion of 8 to 2 in good yield. There seems to be no advantage in the stepwise conversion of 8 to 25 to 2.

Unfortunately, only fresh palladium oxide, finely powdered, gave good results. A more convenient and reliable catalyst system was sought. Palladium chloride catalyzed the reduction of 8 to 2 but the hydrogen chloride liberated by the reduction of the catalyst caused the formation of 25% of isogalbulin (27). The addition of inorganic buffers minimized the formation of 27 and increased the activity of the catalyst, allowing the hydrogenation of 8 to 2 consistently in 65–75% yield. Synthetic 2 was identical with the tetramethyl ether of natural NDGA.

Finally, demethylation of 2 with refluxing concentrated hydrobromic acid afforded, in nearly quantitative yield, NDGA (1), identical with a purified sample of the natural product.

An alternative route to 2 involves reductive ring opening[19,20] of 25 by a solution of sodium in liquid ammonia and tetrahydrofuran to give the alcohol 28, which is probably an intermediate in the hydrogenations of 8 and 25 already described. Cyclization of 28 by a very mild acid treatment[20] gave racemic isogalbulin (27) in good yield. Hydrogenolysis of the acetate 29 gave 2. However, this longer route to 2 seems to have no advantages over the direct hydrogenation of 8.

Experimental Section

Microanalyses were performed by Dr. F. Scheidl and associates of the Hoffmann-La Roche Inc. microchemical laboratory. Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected. Spectra were recorded on standard instruments by the staff of the Physical Chemistry Department of Hoffmann-La Roche Inc., or on a Perkin-Elmer Model 237B Infracord. Unless otherwise noted, pmr spectra were recorded at 60 MHz. Chemical shifts are expressed as δ values (parts per million downfield from tetramethylsilane as internal reference) and coupling constants (J) are expressed in cycles per

(18) We thank Mr. D. Wagner, Hoffmann-La Roche Inc., for assistance with the high-pressure hydrogenation experiments.

(19) A. J. Birch, B. Milligan, E. Smith, and R. N. Speake, *J. Chem. Soc.*, 4471 (1958).

(20) N. S. Crossley and C. Djerassi, *ibid.*, 1459 (1962).

second. Thin layer chromatograms (tlc) were used routinely for following reactions and separations, and were performed on Brinkmann F254 silica gel plates, which were examined under long- and short-wave ultraviolet light and were then sprayed with a 1:1 mixture of 85% phosphoric acid and concentrated nitric acid and warmed gradually on a hot plate. Gas chromatographic analyses were carried out on a 4 ft × 0.25 in. o.d. copper column packed with 1.1% SE-30 + 0.2% Versamid 900 on AW/DMCS Chromosorb G, 60–80 mesh, installed in an instrument equipped with a thermal conductivity detector, using helium as a carrier and operating at 220–250°.

Purification of NDGA (1).—Commercial NDGA[10] was recrystallized three times from 36% aqueous acetic acid to give tan-colored material, mp 183–184°. This was sublimed at 175–180° (0.03 mm) to afford large white and some yellow crystals. The white crystals were hand picked and ground in a mortar, giving pure NDGA: mp 185–186° (lit.[8] mp 184–185°); optically inactive; ν_{max} (KBr) 3470, 3310, 3200, 1612, 1530, 1520, 790, 755 cm^{-1}; nmr (CD_3OD) δ 0.78 (6 H, d, J = 6 Hz), 1.66 (2 H, poorly resolved q, J = 6 Hz), 2.12 (2 H, unsymmetrical q, J = 13, J' = 9 Hz), 2.61 (2 H, unsymmetrical q, J = 12, J' = 6 Hz), 4.98 (4 H, broad s), 6.36–6.75 (6 H, m); λ_{max} (CH_3OH) 283, 218 mμ (ϵ 6660, 13,400).

NDGA Tetramethyl Ether (2) from Commercial NDGA.—Addition of potassium hydroxide solution (16 g of KOH in 50 ml of water and 75 ml of methanol) to a solution of NDGA[10] (20.0 g, 0.0663 mol) in methanol (60 ml) at room temperature under nitrogen gave a dark solution to which dimethyl sulfate (67 g, 0.532 mol) was added with stirring over a period of 15 min. The temperature during the addition was maintained at 35–40°, and enough potassium hydroxide solution was introduced periodically to maintain pH 8–9. The reaction mixture was stirred at room temperature for 18 hr and then poured into water. The precipitate was collected by filtration, washed with water, and dried to provide 22.7 g (96%) of tan-colored solid, mp 93–98°. Purification of the product by elution from Florisil with benzene–chloroform (1:1) followed by several recrystallizations from methanol afforded analytically pure product: optically inactive; mp 100–102° (lit.[8] mp 100–101°); ν_{max} (KBr) 1610, 1595, 1525, 1265, 1240, 1160, 1140, 1025 cm^{-1}; nmr ($CDCl_3$) δ 0.85 (6 H, d, J = 6 Hz), 1.50–3.00 (6 H, m), 3.87 (12 H, s), 6.60–6.84 (6 H, m); λ_{max} (CH_3OH) 279, 228 mμ (ϵ 6080, 16,400).

Anal. Calcd for $C_{32}H_{50}O_4$: C, 73.71; H, 8.44. Found: C, 73.77; H, 8.48.

***meso*-1,4-Bis(2-bromo-4,5-dimethoxyphenyl)-2,3-dimethylbutane (3).**—NDGA tetramethyl ether (2, 5.0 g, 14 mmol) was brominated in acetic acid[9] in 91% yield. After several recrystallizations from methanol the product had mp 131–132°. A sample of large, needlelike crystals, obtained by slow evaporation of a saturated methanol solution, was subjected to X-ray crystallographic examination[11] and found to be the meso isomer.

3,4-Dimethoxypropiophenone (4).—To a cooled, well-stirred slurry of anhydrous aluminum chloride (22.0 g, 0.166 mol) in chloroform (80 ml) at 0–5° under an atmosphere of dry nitrogen was added a solution of freshly distilled propionyl chloride (12.0 g, 0.13 mol) in chloroform (10 ml) at such a rate as to maintain a temperature of 0–5°. When the addition was complete (about 15 min), a solution of veratrole (13.8 g, 0.1 mol) in chloroform (10 ml) was added in the same manner over a 30-min period, during which time hydrogen chloride was slowly evolved. The reaction mixture became a nearly clear, yellow-green solution, which was stirred at 0–5° for 1 hr after completion of the addition. With continued stirring and cooling 3 *N* hydrochloric acid (100 ml) was then added *very* cautiously dropwise, keeping the temperature below 30°. When all the solids were dissolved, the phases were separated, the lower organic phase was washed with 3 *N* sodium hydroxide solution (50 ml) once, and the two aqueous solutions were back-extracted in succession with chloroform (50 ml). The combined chloroform solution was dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness *in vacuo*, and the residue was crystallized from methanol (25 ml) by chilling overnight in a freezer to afford a first crop, 14.31 g (78.8% yield), mp 58.5–59.5°. A second crop, 3.84 g (20.0% yield), had mp 54–58°. An analytical sample had mp 58–59° (lit.[12] mp 59–60°).

α-Bromo-3,4-dimethoxypropiophenone (5).—A solution of bromine (65.4 g, 0.408 mol, 2% excess) in chloroform (100 ml) was added as rapidly as possible through an addition funnel to a refluxing solution of 3,4-dimethoxypropiophenone (4, 77.6 g, 0.40 mol) in chloroform (300 ml) with good agitation. The hydrogen bromide, which was rapidly evolved, was conducted from the top of the reflux condenser to a flowing water scrubber. When the addition was complete the solution was refluxed for 10 min to drive off most of the hydrogen bromide; then the solvent was removed under reduced pressure. Crystallization of the residue from methanol (200 ml) gave a first crop, 101.9 g (93.2% yield), mp 81–82°. A second crop, 2.3 g (2.2% yield), had mp 72–77°, and an analytical sample had mp 82–82.6° (lit.[13] mp 83–84°).

***meso*-2,3-Bis(3,4-dimethoxybenzoyl)butane (7).** A.—The procedure described by Atkinson and Haworth[14] was used, except that commercial copper powder was used in place of "freshly precipitated copper"[21] and a small crystal of iodine was added to the reaction mixture. Crystallization of the crude product from methanol afforded white crystals: mp 184.5–187.5° (lit.[14] mp 189–190°); 3.8% yield; nmr ($CDCl_3$) 1.15 (6 H, d, J = 6 Hz), 4.01 (14 H), 6.90–8.0 (6 H, m); *m/e* (rel intensity) 386 (10), 221 (2), 194 (2), 180 (2), 165 (100), 137 (3), 122 (2).

B.—α-Bromo-3,4-dimethoxypropiophenone (5, 2.73 g, 10 mmol), copper powder (2.73 g, 43 mmol), benzene (20 ml), and diphenyl ether (20 ml) were stirred together under a nitrogen atmosphere and solvent was distilled until the reaction temperature reached 150°. The reaction mixture was stirred at 150° for 18.5 hr; then it was filtered and the filtrate was chromatographed on activity I neutral alumina. Diphenyl ether was eluted with benzene, the crude diketone was eluted with benzene–ether (19:1 through 1:1) and more polar products were eluted with ethyl acetate. Crystallization of the diketone fraction gave fine white needles of 7, mp 186–190° (137 mg, 7.1% yield). Treatment of the mother liquors plus the ethyl acetate eluates with boiling ethanolic hydrochloric acid gave crude crystals of 8, mp 169.5–170.5° (lit.[14] mp 169–170°), 376 mg (20.4% yield).

3,4-Dimethyl-2,5-bis(3,4-dimethoxyphenyl)furan (8). A.—A solution of *meso*-2,3-bis(3,4-dimethoxybenzoyl)butane (7, 24 mg) in 10% ethanolic hydrochloric acid (5 ml) was refluxed for 15 min and cooled to provide colorless crystals of 8, mp 169–170.5° (lit.[14] mp 169–170°), 20 mg (87.4% yield).

B.—To a boiling solution of *rac*-2,3-bis(3,4-dimethoxybenzoyl)-butane (9, 38.6 g, 0.10 mol) in dichloromethane (100 ml) was added a 1% solution of hydrogen chloride in methanol (250 ml) slowly with continued boiling. After about 5 min crystals separated, and after the slurry was chilled a first crop was obtained, mp 170–171°, 30.20 g (82% yield). Concentration of the mother liquors afforded a second crop, mp 169.5–170.5°, 4.56 g (12.4% yield), and a third crop, mp 168.2–170.0°, 0.54 g (1.5% yield).

Racemic 2,3-Bis(3,4-dimethoxybenzoyl)butane (9).—To liquid ammonia (approximately 50 ml) was added powered ferric chloride (50 mg), then small pieces of sodium (0.51 g, 0.022 g-atom, 10% excess) were added and the blue color was allowed to dissipate over about a 20-min period. To the resulting gray suspension of sodamide was added solid 3,4-dimethoxypropiophenone (4, 3.88 g, 0.02 mol) in small portions and the mixture was stirred for about 5 min. Solid α-bromo-3,4-dimethoxypropiophenone (5, 5.46 g, 0.02 mol) was then added in small portions to the gray-green mixture, and the reaction mixture turned deeper green, then reddish, and finally tan colored. After the mixture was stirred for 1 hr, solid ammonium chloride (2.68 g) was added, followed by dichloromethane (50 ml), and the gray mixture was then warmed cautiously to room temperature to evaporate most of the ammonia. The mixture was filtered with suction, the residual solids were extracted twice with dichloromethane, and the combined filtered solutions were concentrated to about 50 ml in volume, diluted with methanol (75 ml), and further concentrated to about 50 ml in volume by boiling. The product crystallized on stirring and cooling, mp 137–141°, 6.96 g (90.3% yield). Recrystallization from methanol provided an analytical sample: mp 145–146°; ν_{max} (KBr) 3080, 1665, 1595, 1585, 1515, 1265, 1245 cm^{-1}; nmr ($CDCl_3$) δ 1.33 (6 H, d, J = 7 Hz), 3.92 (14 H, "d"), 6.9–8.0 (6 H, m); mass spectrum *m/e* 386 (11), 221 (2), 194 (<1), 180 (<1), 165 (100), 137 (3), 122 (4).

Anal. Calcd for $C_{32}H_{36}O_8$: C, 68.37; H, 6.78. Found: C, 68.16; H, 6.83.

Base-Catalyzed Equilibration of Diketones 7 and 9.—A mixture of 7 (50 mg), methanol (20 ml), benzene (5 ml), ethanol (20 ml), and sodium methoxide (62 mg) was boiled gently for about 1 hr and then stored at room temperature overnight. The solution

(21) In one experiment "freshly precipitated copper" was used, but only traces of 7 could be detected in the product.

was boiled down to a volume of 15 ml, powdered magnesium sulfate (100 mg) was added to neutralize base, the mixture was filtered, and the filtrate was reduced to dryness under reduced pressure. Extraction of the residue twice with chloroform and removal of the solvent left a residue (48 mg) which was analyzed by nmr spectrometry (100 MHz), using the signals at δ 1.15 ppm (compound 7) and 1.33 ppm (compound 9) to determine the ratio of 9:7, which was 1.87.

Similarly, 9 (50 mg) gave a mixture (48 mg) with a ratio of 9:7 = 2.17.

α-Chloro-3,4-dimethoxypropiophenone (6).—A solution of α-chloropropionyl chloride (50.1 g, 0.39 mol) in chloroform (50 ml) was added to a stirred slurry of anhydrous aluminum chloride (66.5 g, 0.50 mol) in chloroform (300 ml), while the temperature of the mixture was maintained at −5 to 0°. A solution of veratrole (41.4 g, 0.30 mol) in chloroform (40 ml) was then added dropwise while the same temperature was maintained. The dark solution was stirred for 4 hr at 0–5°, then for 1 hr at room temperature. Hydrochloric acid (3 *N*, 300 ml) was added very cautiously with ice-bath cooling, and the mixture was extracted and washed as described for compound 4 to afford 55 g of dark brown liquid. Column chromatography over activity I neutral alumina (600 g) afforded a fraction (6.47 g, eluted with hexane–benzene and benzene–chloroform mixtures) which solidified. Crystallization from hexane–benzene gave colorless needles (6.4 g, mp 57–60°). An analytical sample had mp 58.5–60.5°, ν_{max} (KBr) 1692 cm^{-1}.

Anal. Calcd for $C_{11}H_{13}ClO_3$: C, 57.78; H, 5.73; Cl, 15.50. Found: C, 57.80; H, 5.47; Cl, 15.41.

2,4-Dimethyl-3,5-bis(3,4-dimethoxyphenyl)furan (11).—A solution of the enolate of 4 (3.88 g, 0.02 mol) was prepared in 75 ml of liquid ammonia as described above. The chloro compound 6 (4.57 g, 0.02 mol) was added rapidly and the reaction mixture was stirred for 5 hr. After addition of solid ammonium chloride (1.2 g) and dichloromethane (50 ml), the ammonia was allowed to evaporate. Extraction with dichloromethane gave a green, viscous liquid (8.7 g), the hexane-insoluble fraction of which was triturated with methanol to give a solid (0.935 g, mp 154–157°). Recrystallization from methanol afforded an analytical sample: mp 158–160°; λ_{max} (MeOH) 290 nm (ϵ 26,830), inflection 233 (18,950); nmr ($CDCl_3$) δ 2.17 (3 H, s), 2.35 (3 H, s), 3.90 and 3.95 (6 H, 2 s), 7.0 (6 H, m).

Anal. Calcd for $C_{22}H_{24}O_5$: C, 71.72; H, 6.57. Found: C, 71.87; H, 6.54.

3,4-Dimethoxyacetophenone (13).—By the same procedure used for the preparation of 4, veratrole was acetylated to 13 in 82% yield, mp 48–50° (from benzene–hexane) (lit.[22] mp 48°).

α-Bromo-3,4-dimethoxyacetophenone (14).—Treatment of 13 (18.0 g, 0.10 mol) in chloroform (75 ml) with a solution of bromine (16.3 g, 0.102 mol) in chloroform (25 ml) at 25° gave 16.5 g (64% yield) of crude 14. An analytical sample recrystallized from benzene–hexane had mp 80–81° (lit.[23] mp 80–81°).

1,2,3-Tris(3,4-dimethoxybenzoyl)propane (15).—A suspension of sodamide in liquid ammonia (50 ml) was prepared from sodium (0.51 g, 0.022 mol), and 13 (3.60 g, 0.02 mol) was added in small portions, followed by 14 (5.18 g, 0.02 mol). The mixture was stirred at −33° for 3 hr and worked up in the usual manner to afford 10 g of dark, viscous liquid, which was chromatographed over 250 g of neutral alumina. Several fractions solidified and were combined and crystallized from benzene–hexane to afford 1 g of 15: mp 174–175°; ν_{max} (KBr) 1680 cm^{-1} (broad); λ_{max} (CH_3OH) 229, 276, 306 nm (ϵ 49,000, 33,520, 27,310); nmr ($CDCl_3$) 3.39 (4 H, q, J = 7, J' = 3 Hz), 3.88 and 3.93 (18 H, 2 s), 4.75 (1 H, t, J = 7 Hz), 6.8–8.0 (9 H, m); mass spectrum *m/e* 165 (base peak), 370 (M − ArCO, H), 536 (M^+).

Anal. Calcd for $C_{30}H_{32}O_9$: C, 67.15; H, 6.01. Found: C, 67.11; H, 6.01.

***trans*-1,2,3-Tris(3,4-dimethoxybenzoyl)cyclopropane (16).**—Fractional crystallization from the mother liquors of 15 afforded 150 mg of a second compound, 16: mp 202–204°; ν_{max} (KBr) 1660 cm^{-1} (broad); λ_{max} (CH_3OH) 230, 280, 312 nm (ϵ 33,400, 24,530, 23,940); nmr ($CDCl_3$) δ 3.7–4.3 (21 H, m, with three strong singlets for $-OCH_3$), 6.8–8.1 (9 H, m); mass spectrum *m/e* 165 (base peak), 369 (M − ArCO), 534 (M^+).

Anal. Calcd for $C_{30}H_{30}O_9$: C, 67.40; H, 5.66. Found: C, 67.51; H, 5.61.

Racemic 2,3-Dimethyl-1,4-diphenyl-1,4-butanedione (22).—Propiophenone (13.8 g, 0.1 mol) was added to a suspension of sodamide (0.13 mol) in liquid ammonia (200 ml) and stirred for 15 min before addition of α-bromopropiophenone (21.3 g, 0.1 mol). The reaction mixture was stirred for 30 min and the ammonia was replaced with methylene chloride. Extraction with methylene chloride gave 26 g of dark-colored liquid which was chromatographed over 100 g of Florisil. Elution with hexane–benzene mixtures gave a solid which after recrystallization from hexane weighed 10.0 g: mp 86–87°;[24] ν_{max} (KBr) 1680 cm^{-1}; nmr ($CDCl_3$) δ 1.27 (6 H, d, J = 6 Hz), 3.75 (2 H, m), 7.2–8.0 (10 H, m); mass spectrum *m/e* 266 (M^+).

Anal. Calcd for $C_{18}H_{18}O_2$: C, 81.17; H, 6.81. Found: C, 80.90; H, 6.89.

Boiling 22 in methanolic hydrogen chloride for a few minutes converted it to 2,5-diphenyl-3,4-dimethylfuran, mp 114–115° (lit.[25] mp 116°), in good yield.

α-Bromo-*p*-methoxypropiophenone (21).—Bromination of *p*-methoxypropiophenone in refluxing chloroform provided 21 in 86% yield, mp 66–69° (from methanol) [lit.[26] mp 68.5° (petroleum ether, bp 30–60°)].

Racemic 2,3-Bis(*p*-methoxybenzoyl)butane (23).—The condensation of *p*-methoxypropiophenone (16.4 g, 0.10 mol) with 21 (24.3 g, 0.10 mol) was carried out as described for compound 22 above. The product was crystallized from methanol to afford a cream-colored solid (22.1 g, 67% yield), mp 116–121°. An analytical sample melted at 124–127°: ν_{max} (KBr) 1660 cm^{-1}; nmr ($CDCl_3$) δ 1.30 (6 H, d, J = 6 Hz), 3.9 (8 H, strong s over m), 7.0 and 8.1 (8 H, aromatic AA′BB′ pattern); mass spectrum *m/e* 135 (base peak), 326 (M^+).

Anal. Calcd for $C_{20}H_{22}O_4$: C, 73.60; H, 6.79. Found: C, 73.80; H, 6.64.

***all-cis*-3,4-Dimethyl-2,5-bis(3,4-dimethoxyphenyl)tetrahydrofuran (25).**—Hydrogenation of the furan 8 (36.8 g, 0.1 mol) over 10% palladium on calcium carbonate catalyst (5.0 g) in ethanol (1000 ml) at 125° under 1500 psig hydrogen for 3 hr, followed by filtration and removal of solvents, gave a white solid (35 g) which on recrystallization from methylene chloride–methanol gave a first crop, mp 127.5–130.0° (29.86 g, 80.4% yield). Concentration of the mother liquors gave a second crop, mp 125.5–128.0° (2.54 g, 6.8% yield), and a third crop, mp 124.5–127.5° (0.47 g, 1.3% yield). An analytical sample had mp 131–132° (lit.[8] mp 132–133°); nmr ($CDCl_3$) δ 0.62 (6 H, d, J = 7 Hz), 2.70 (2 H, m), 3.94 (12 H, s), 5.19 (2 H, d, J = 6.5 Hz), 7.0–7.17 (6 H, m).

Hydrogenations of 8 and 25. General Procedure.—A mixture of the compound to be reduced (0.2–3.68 g), catalyst (usually 0.2 g), and solvent (50 ml) was stirred magnetically under an atmosphere of hydrogen at the desired temperature. After filtration of the spent catalyst, the filtrate was examined by gas chromatographic analysis. For isolation of crude NDGA tetramethyl ether (2), the solvents were removed and the residue was crystallized from 10–20 times its weight of hexane.

NDGA Tetramethyl Ether (2). A. From 8.—The furan 8 (33.3 g, 90.5 mmol) in THF (500 ml) was hydrogenated over powdered palladium oxide (2.0 g) at 50° (1500 psig) for about 10 hr. Gas chromatographic analysis indicated 77.8% of 2 in the crude filtrate. Removal of solvent and crystallization of the residue (33.7 g) from hexane (550 ml) gave crude 2, mp 91.5–95° (25.4 g, 78% yield).

B. From 25.—Compound 25 (745 mg; 2 mmol) in THF (50 ml) was hydrogenated over powdered palladium oxide (200 mg) at 25° (1 atm) for 46 hr. Gas chromatographic analysis indicated 78.5% of 2 in the crude filtrate. Removal of solvent and crystallization of the residue (848 mg) from hexane gave crystalline 2, mp 93–95° (543 mg, 76% yield).

C. From 8, Using Palladium Chloride.—The furan 8 (4.00 g), palladium chloride (0.40 g), and sodium acetate (0.46 g) in tetrahydrofuran (100 ml) were shaken in a rocking autoclave at 75° under hydrogen at 50 psig for 10 hr. Gas chromatography indicated a yield of 79% of 2, 85% of which could be isolated as described above.

NDGA (1) from 2.—Concentrated hydrobromic acid (860 g) was added under nitrogen to 2 (71.56 g, 0.201 mol) and the mixture was stirred and refluxed for 9 hr and allowed to cool to room

(22) H. Stephen and C. Weismann, *J. Chem. Soc.*, 105, 1046 (1914).
(23) C. Mannich and F. L. Hahn, *Ber.*, 44, 1542 (1911).
(24) The meso isomer melts at 67°: A. Wolf, German Patent 876,237 (May 11, 1953); *Chem. Abstr.*, 53, 9227*b* (1958).
(25) R. E. Luts and R. J. Taylor, *J. Amer. Chem. Soc.*, 55, 1593 (1933).
(26) C. Hell and A. Hollenberg, *Ber.*, 682 (1896).

temperature overnight with continued stirring. The dark-colored solid product, collected by filtration, washed with water, and dried, had mp 174–182° (59.27 g, 97.5% yield). Recrystallization from about 1600 ml of 20% aqueous acetic acid with charcoal treatment gave a much lighter colored, but still gray-brown, crystalline product, mp 184–186° (45.74 g) (77% recovery, 75% yield). A second recrystallization with charcoal gave light tan crystals, mp 184–185.3° (91% recovery). After a third recrystallization the cream-colored crystals (95% recovery) had mp 184.5–186°, identical in all respects with purified natural NDGA, mmp 184–186°.

The overall yield of thrice-recrystallized product was 65%. No product of satisfactory quality could be recovered from the mother liquors.

Monoalcohol 28.—Sodium (4.37 g, 0.19 mol) was added to anhydrous liquid ammonia (1.1 l.) and stirred under reflux under a nitrogen atmosphere. After 1 hr a solution of the tetrahydrofuran **25** (18.6 g, 50 mmol) in THF (500 ml) was added. After 2.5 hr of stirring under reflux the ammonia was removed by warming the reaction mixture to room temperature. Addition of methanol (10 ml) and then water (400 ml), followed by extraction with chloroform, washing with water, drying over sodium sulfate, and removal of solvents, gave the crude product (22.0 g), which was crystallized from methanol to afford white crystals of **28**, mp 107.5–109° (16.34 g, 43.7 mmol, 87.4% yield). A second crop was collected to give a total of 17.96 g (48 mmol, 96% yield). An analytical sample was recrystallized from benzene–hexane: mp 110–111.3°; ν_{max} (KBr) 3570, 1258, 1238, 1135, 1130, 1025 cm^{-1}; λ_{max} (CH_3OH) 229, 269, 285 mμ (ε 16,553, 5824, 4883); nmr ($CDCl_3$) δ 0.84 (3 H, d, J = 6 Hz), 1.10 (3 H, d, J = 6 Hz), 1.80 (2 H, m), 1.98 (1 H, broad s, exchangeable with D_2O), 2.10 (1 H, q, J_1 = 13, J_2 = 10 Hz), 2.82 (1 H, q, J_1 = 13, J_2 = 3 Hz), 3.70–4.17 (12 H = 4 CH_3O), 4.70 (1 H, d, J = 6 Hz), 6.40–7.00 (6 H, m).

Anal. Calcd for $C_{32}H_{30}O_8$: C, 70.56; H, 8.08. Found: C, 70.74; H, 7.96.

Racemic Isogalbulin 27.—A slurry of **28** (2.52 g, 6.72 mmol) in ethanol (18 ml) was treated with concentrated hydrochloric acid (3 ml) and stirred for 4.5 hr at room temperature. The solution was poured into water and extracted with ether to give a crude product (2.84 g) which was crystallized from methanol with cooling. Recrystallization from hexane provided 1.71 g (4.8 mmol, 72.4%) of **27** as a white solid, mp 66.5–72°. An analytical sample was recrystallized successively from hexane, ethanol, and methanol: mp 70.5–72.5° (lit.[19] mp 86°); ν_{max} (KBr) 1470, 1262, 1250, 1150, 1140, 1109 cm^{-1}; λ_{max} (CH_3OH) 204, 232, 282, 287 mμ (ε 62,750, 16,500, 7100, 6600); nmr ($CDCl_3$) δ 0.97 (6 H, d, J = 7 Hz), 2.5 (4 H, m), 3.67–4.10 (13 H, m), 6.71 (5 H, m).

Anal. Calcd for $C_{31}H_{28}O_4$: C, 74.13; H, 7.92. Found: C, 73.86; H, 7.83.

Monoacetate 29.—To an ice-cold mixture of acetic anhydride (10 g) and pyridine (10 g) was added **28** (3.74 g, 10 mmol) and the mixture was stirred for 5 min before being allowed to warm to room temperature. After 2 hr the mixture was poured into water (200 ml), extracted with chloroform, washed with water, dried, and freed of solvent. The crude product (4.59 g) was crystallized from methanol to give 671 mg of starting material. Second and third crops gave a total of 2.75 g (6.62 mmol, 80.6% corrected yield) of **29**, mp 77–82°.

An analytical sample was recrystallized from hexane: mp 80–82°; ν_{max} (KBr) 1760, 1270, 1240, 1155, 1135, 1030 cm^{-1}; λ_{max} (CH_3OH) 229, 278, 285 mμ (ε 14,100, 4850, 3900); nmr ($CDCl_3$) δ 0.80 (3 H, d, J = 6 Hz), 1.07 (3 H, d, J = 6 Hz), 2.07 (3 H, s), 2.50 (4 H, m), 3.78–3.90 (12 H = 4 OCH_3), 5.80 (1 H, d, J = 8 Hz), 6.71 (6 H, m).

Anal. Calcd for $C_{34}H_{32}O_9$: C, 69.21; H, 7.74. Found: C, 69.48; H, 7.82.

All subsequent experiments gave a dimorphic form of **29**, mp 100–102°, the solution spectral properties of which were identical with those of the lower melting acetate. The lower melting dimorph was converted to the higher melting one by recrystallization from hexane and seeding with the higher melting solid. However, conversion of the higher melting to the lower melting dimorph could not be achieved.

Hydrogenolysis of 29.—A mixture of **29** (1.045 g, 2.5 mmol), ethyl acetate (50 ml), and powdered palladium oxide (100 mg) was hydrogenated at room temperature and 1 atm for 22 hr. Filtration and removal of solvent gave 1.015 g of oil which was crystallized from hexane to give 0.703 g (1.96 mmol, 79%) of crude **2** as a white solid, mp 93–95.5°. Gas chromatographic analysis of the sample showed that it contained 90.3% **2** and 9.7% **27**.

Registry No.—**1**, 27686-84-6; **2**, 24150-24-1; **3**, 36287-35-1; **6**, 36287-36-2; **7**, 36287-37-3; **9**, 27686-81-3; **11**, 36287-39-5; **15**, 36287-40-8; **16**, 36287-41-9; **22**, 36287-42-0; **23**, 36208-08-9; **25**, 27686-82-4; **27**, 36286-72-3; **28**, 36286-73-4; **29**, 36286-74-5.

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ARTICLES

New Soluble-Formazan Assay for HIV-1 Cytopathic Effects: Application to High-Flux Screening of Synthetic and Natural Products for AIDS-Antiviral Activity

*Owen S. Weislow, Rebecca Kiser, Donald L. Fine, John Bader, Robert H. Shoemaker, Michael R. Boyd**

We have developed an effective and optimally safe microculture method for rapid and convenient assay of the in vitro cytopathic effects of human immunodeficiency virus (HIV-1) on human lymphoblastoid or other suitable host cells. The assay procedure is applicable to the evaluation of drug effects on in vitro infections induced directly in cultured host cells by cell-free HIV-1 or by coculture with H9 cells chronically infected with HIV-1. The assay uses a newly developed tetrazolium reagent that is metabolically reduced by viable cells to yield a soluble, colored formazan product measurable by conventional colorimetric techniques. This simple microassay minimizes the number of plate manipulations typically required with other assay methods and, coupled with computerized data collection and analysis, facilitates large-scale screening of agents for potential antiviral activity. To support and enhance the discovery of new anti-HIV-1 agents, the National Cancer Institute is offering investigators worldwide the opportunity to submit new candidate agents for anti-HIV-1 screening with this method. [J Natl Cancer Inst 81:577-586, 1989]

Screening is an essential tool for new drug discovery. The use of a relatively simple in vitro assay was critical to the early identification of 3'-azido-3'-deoxythymidine (AZT), the first new antiviral drug with demonstrable therapeutic efficacy in acquired immunodeficiency syndrome (AIDS) (*1,2*). However, since the discovery of AZT, relatively few new nonnucleoside classes of compounds that interfere with replication and/or cytopathic effects of human immunodeficiency virus (HIV-1) have been discovered. Current antiviral screening strategies typically depend on analysis of viral antigen synthesis (*3,4*) or reverse transcriptase activity (*5*). Although these methods are sensitive, they involve relatively lengthy procedures and do not simultaneously provide a quantitative measure of drug cytotoxicity that would allow estimation of an in vitro "therapeutic index." Techniques that do provide direct, quantitative determination of HIV-1-induced cytopathic effects typically require time-consuming macroscopic observation of plaque formation (*6*) or microscopic observation of cell viability (*7,8*) or syncytium formation (*9,10*). Consequently, these techniques may not be useful for large-scale screening of potential antiviral compounds.

As part of the response of the National Institutes of Health (NIH) to the AIDS epidemic, the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (NCI), has undertaken the development of a project for large-scale drug screening that would allow greatly expanded testing of synthetic and natural products for anti-HIV-1 activity (*11*). This screening program is intended to serve as a national resource, permitting scientists from academic or industrial settings worldwide to submit novel compounds for screening. The screen will also be used extensively to search for new potential anti-HIV-1 compounds from the NCI repositories of synthetic and natural

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O. S. Weislow, R. Kiser, and D. L. Fine, Research Support Program, Program Resources, Inc., National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD.

J. Bader, R. H. Shoemaker, and M. R. Boyd, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD.

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**Correspondence to:* Michael R. Boyd, M.D., Ph.D., Office of the Associate Director for Developmental Therapeutics, Division of Cancer Treatment, National Cancer Institute, Executive Plaza North, Room 843, Bethesda, MD 20892.

products. Establishment of a high-flux drug screen with the potential to accommodate as many as 40,000 or more samples per year has required the development of a simple, automated method consistent with procedures for safe handling of HIV-1.

Assays based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) have been used to measure virus-induced cytopathic effects (*12,13*) and cell proliferation (*14*). The cellular reduction of MTT results in the formation of a colored, insoluble formazan product. For quantitative estimation of cell growth and viability, the formazan must be solubilized prior to colorimetric determination. For nonadherent cell preparations, centrifugation may also be required. We describe a new, simplified, tetrazolium-based assay that we have derived from methods recently developed for in vitro antitumor screening (*15,16*). This assay uses the new tetrazolium reagent 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) and cell lines sensitive to the lytic effects of replicating HIV-1. The assay detects drug-induced suppression of viral cytopathic effects by generation of a soluble formazan (XTT formazan) (fig. 1) in surviving cells.

Materials and Methods

Cells and Viruses

CEM-SS cells were obtained from Peter Nara (*10*); MT-2 cells transformed with HTLV-I (*6*), from Douglas Richmond; C3-44 cells transformed with HTLV-II, from William Mitchell (*17*); and LDV-7 cells, from Robert Gallo. The suitability of various T-lymphocyte-derived cell lines for inclusion in the microculture XTT antiviral assay was initially determined on the basis of their sensitivity to the lytic effects of HIV-1 infection. Other criteria were the abilities of the cells to metabolically reduce XTT to measurable quantities of XTT formazan and to show increased XTT formazan production due to inhibition of virus infection by an agent. More recently, cell lines have also been evaluated for use on the basis of their relative ease of growth; for example, the ability to grow in the absence of interleukin-2. All cells are grown in RPMI-1640 medium plus 10% fetal calf serum with 50 μg of gentamicin/mL. To ensure consistency of target-cell sensitivity to virus and drug, cells are routinely replaced every 2 months from frozen stocks. Similarly, virus-producing H9 cells used in the cocultivation (cell-to-cell) assay described here are discarded and replaced with freshly infected cells every 2 months.

Virus stocks were prepared in H9 cells. The viability of chronically infected H9 cell cultures and their capacity to produce high virus titers were enhanced by (*a*) maintenance of the cultures in the exponential phase of growth and (*b*) periodic addition of log-phase, uninfected H9 cells. In the standard procedures for the maintenance of H9 cells, we used our modification of a protocol suggested by P. Nara (personal communication). Once a week, we divided cultures of H9 cells that were producing HIV-1 (III_b or RF variants). We diluted stock cell cultures 1:5 or 1:10 to yield a final concentration of 1×10^5 cells/mL. At the time the cultures were divided, one-fifth of the volume of infected cells was replaced with an equal volume of uninfected cells. Three days after the split, 20% of the medium was replaced with fresh medium, and 2 days after the addition of uninfected cells, virus-containing supernatants were harvested by centrifugation at 3,000 rpm for 15 minutes at 4 °C. Aliquots (1 mL) of the supernatants were frozen in liquid nitrogen for later use. We titrated virus stocks using a 4-day syncytium assay as previously described (*10*). Highly infectious, virus-producing H9 cells were maintained for the cocultivation assay by daily 50:50 dilution of the cultures described here in fresh medium. The syncytium assay of Nara et al. (*10*) was used for quantitation of the number of virus-producing cells in the infected H9 culture; 80%–90% of these cells were producing virus. Typical concentrations of infectious virus are 3×10^4 syncytium-forming units (SFU)/mL for the III_b variant of HIV-1 and 5×10^5 SFU/mL for the RF variant.

Sensitivity to the lytic effects of HIV-1 infection was determined in preliminary experiments for each host cell line by titrations of cell-free virus or H9 cells chronically infected with HIV-1. For the cocultivation studies, the final VIC (quantity of virus-infected cells added) was the lowest number of infected cells that yielded at least 70% uniform suppression of XTT formazan production in host cells in 7 days. This was accomplished with a VIC of 400 infected H9 cells per well in CEM-SS or LDV-7 hosts and 100 infected H9 cells per well in MT-2 or C3-44 hosts. For the studies of cell-free virus infections, the amount of virus added

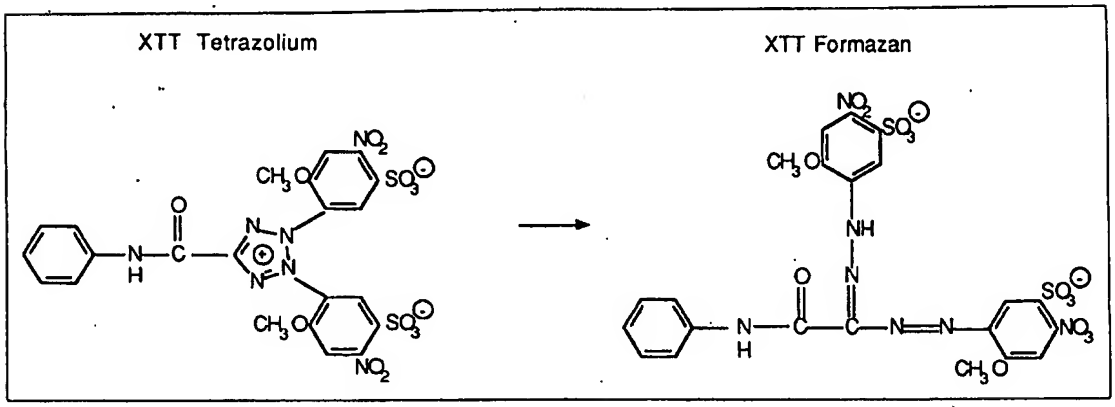

Figure 1. Chemical structures of XTT (colorless) and XTT formazan (orange).

was such that the final multiplicity of infection (MOI; ratio of number of infectious virus particles/number of target cells) was the lowest MOI yielding 70% suppression of XTT formazan production in host cells in 7 days. In the cell-free virus assays, the MOIs, which were established by syncytium analysis, were 0.1 for CEM-SS and LDV-7 hosts and 0.01 for MT-2 and C3-44 hosts.

Drugs

All compounds were provided by the Developmental Therapeutics Program, Division of Cancer Treatment, NCI. Stock solutions were prepared in 100% dimethyl sulfoxide at the highest achievable concentration for each agent. Initial drug dilutions (1:400) resulted in a maximum culture concentration for dimethyl sulfoxide of 0.25%, which had no apparent direct toxic effects on the cell lines used or the HIV-1 infection. Compounds used in these studies included AZT; 2',3'-dideoxyadenosine; 2',3'-dideoxyinosine; 2',3'-dideoxycytidine; dextran sulfate; castanospermine; pepstatin; aurintricarboxylic acid; suramin; and cyclosporine.

XTT Assay

We performed extensive evaluation of two antiviral screening protocols (fig. 2) derived from methods recently described for antitumor assays with XTT (*15*). Our protocols were developed from preliminary evaluations of cell growth characteristics in microtiter trays; virus sensitivities; and drug-dilution protocols (data not shown). These initial studies were required to ensure that there would be sufficient time during the test (*a*) for the virus to kill the target cells or otherwise modulate cell growth, (*b*) for the anti-HIV-1 compound to be effective, and (*c*) for determination that the growth of the cells, and thus XTT formazan production, which is read as optical density, would not exceed the measurement limits for the photometers used.

Figure 2A illustrates the protocol that uses chronically infected H9 cells as the virus source. Infectivity of the H9 line was maintained by periodic dilution of the infected culture with uninfected log-phase H9 cells as described here. Uninfected host cells are mixed with different numbers of HIV-1-infected or uninfected H9 cells and dispensed in 100-μL aliquots to appropriate wells of a microtiter tray containing 100-μL dilutions of test compounds or media. Seven days after distribution of infected cells to the microtiter trays, we add to each well 50 μL of a mixture of 1 mg of XTT/mL and 0.01–0.02 m*M* *N*-methylphenazonium methosulfate (*15*). Details of the synthesis of XTT have been reported previously (*16*), and the reagent is now commercially available (Polysciences, Warrington, PA). The trays are reincubated for an additional 4 hours to allow for XTT formazan production; their plastic covers are then replaced with adhesive plate sealers (Dynatech, Alexandria, VA), the contents of each plate are mixed, and optical densities are determined with a V-max photometer (Molecular Devices, Inc.) at a test wavelength of 450 nm and a reference wavelength of 650 nm. Uninfected cells or cells that are protected by drugs and have continued to proliferate produce the soluble orange XTT formazan (fig. 3A), and the cultures yield high optical densities (ODs). Cells not protected by drugs are killed by the virus and/or do not proliferate; they produce less XTT formazan and thus yield lower optical densities. Data are expressed as percent of XTT formazan from untreated control cells, as determined by the following equation: % of untreated control XTT formazan = (test OD/control cell OD) × 100.

Drugs are further compared on the basis of the concentration that increases XTT formazan production in infected cultures to 50% of that in untreated, uninfected control cells (EC_{50}; protection in infected cells) or the concentration that inhibits XTT formazan production in uninfected cultures to 50% of that in untreated, uninfected control cells (IC_{50}; cyto-

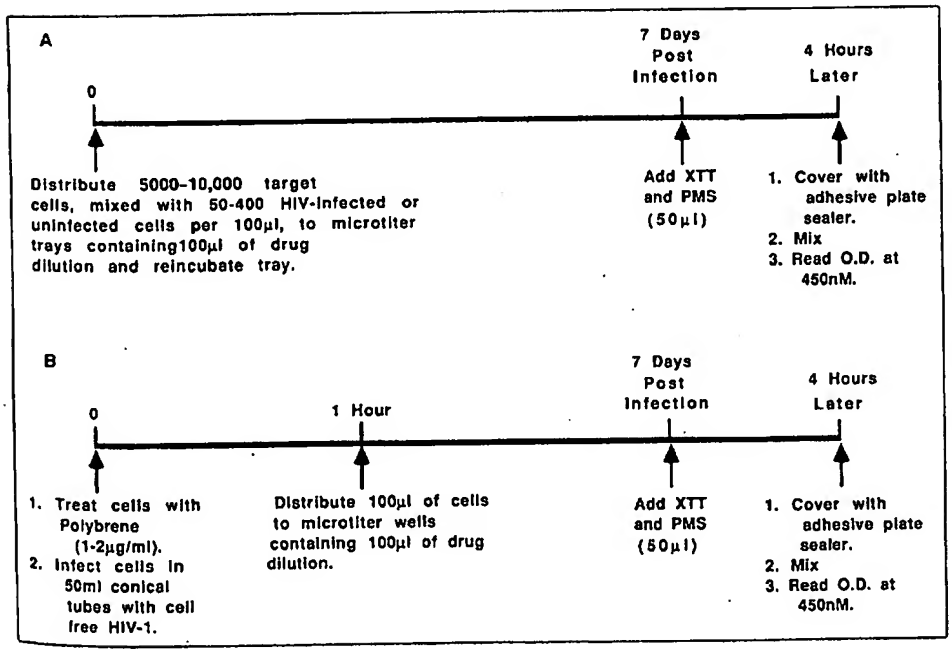

Figure 2. Time lines of critical events in drug-screening protocols. 10^4 target cells infected with cell-free virus (A) or 400 H9 cells infected with HIV-1 (B) are added to dilutions of test compounds in 96-well trays. Trays are incubated at 37 °C in air plus 5% CO_2 for 7 days. A mixture of XTT and *N*-methylphenazonium methosulfate (PMS) is added at the end of this incubation, and plates are reincubated for 4 hr to permit color development (XTT formazan production) as described in Materials and Methods.

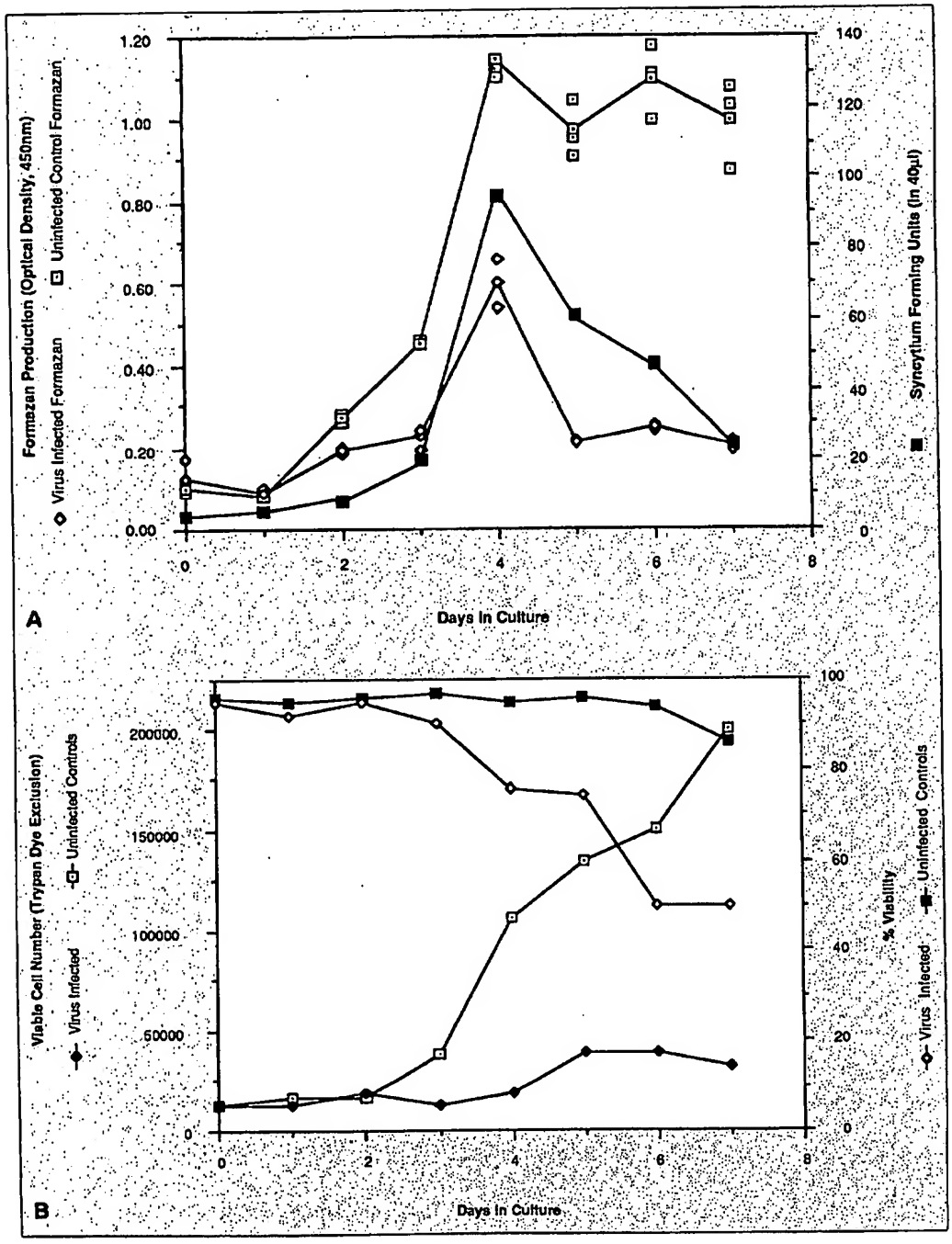

Figure 3. Relationships of XTT formazan production to cell viability and synthesis of infectious virus. CEM-SS cells (10^4) were mixed with H9 cells (400/well) infected with HIV-1 ($IIII_b$) or uninfected. Samples were taken from triplicate wells for assays of XTT formazan and infectious virus at 24-hr intervals for 7 days (A). Samples were also taken at these intervals for trypan blue dye exclusion analysis of cell viability (B) as described in Materials and Methods.

toxicity in uninfected cells). These calculations are performed by simple linear interpolation.

Figure 2B illustrates the procedures used for cell-free virus infection and drug screening. Following pretreatment with 1-2 µg of hexadimethrine bromide (Polybrene)/mL, pelleted cells are incubated in 100-200 µL of medium with cell-free virus for 1 hour in 50-mL conical centrifuge tubes at 37 °C in air plus 5% CO_2. Infected cells are distributed in 100-µL aliquots (containing 10^4 cells) to round-bottom microtiter trays containing, when required, 100 µL of diluted test agents or media; they are then reincubated. After 7 days of incubation, the assay is completed as described for the cocultivation assay.

Syncytium and p24 Assays

We accomplished correlation of XTT formazan production with HIV-1 internal core p24 antigen synthesis using a p24 antigen-capture assay (Du Pont Co., Wilmington, DE). Culture fluids harvested from drug test plates or other sources were diluted 1:100 in 10% triton X-100 and stored frozen at −70 °C until they were required for assay. Aliquots (200 µL) of the triton-treated samples were added in duplicate to microtiter wells previously coated with rabbit polyclonal anti-HIV-1 p24 serum and incubated at room temperature overnight. After washing and blotting, 100 µL of biotinylated polyclonal anti-HIV-1 p24 was added to each of the

appropriate wells, and the plates were reincubated at 37 °C for 60 minutes. A solution of streptavidin-horseradish peroxidase was added after additional washing and blotting. The contents of each plate were mixed and reincubated for 15 minutes at room temperature and, after washing and blotting, the *o*-phenylenediamine dihydrochloride substrate was added in 100-μL aliquots. Color was allowed to develop in the dark at room temperature for 30 minutes, and the reaction was halted by the addition of 4 N H_2SO_4. Optical density was measured at 490 nm, and the concentration of p24 was determined by comparison with a standard curve of known concentrations of p24.

The syncytium assay described by Nara et al. (*10*) was used for quantitation of infectious virus. Supernatants from test plates (40 μL) were examined in CEM-SS cell monolayers at multiple dilutions to obtain countable levels of SFU (50-200 SFU/well) in 4 days. Data were expressed as SFU/mL. Cell-free virus stocks were also titrated by this method.

Quality Controls

The screening of diverse compounds at the rate of 800 or more per week requires effective and efficient data accrual, storage, display, and analysis. Each screening test must be evaluated for uniformity and consistency with respect to appropriate quality controls. It is essential to promptly detect and correct problems that could have arisen in cell growth, virus-induced cytopathic effects, and positive controls. We have incorporated a number of quality control checks that permit computerized monitoring of all data for such potential aberrances. Manual overrides are available, and all data are briefly scanned by the laboratory staff to ensure that quality control standards are met. Although drugs are assayed in duplicate in the test plates, valid statistical data obtained in preliminary studies with three to six replicate wells per point indicate that well-to-well variation in the microculture XTT antiviral assay is generally ≤10%. As a quality control measure, plates are discarded if this variation is >25%. Plates are also discarded if virus-induced cell killing decreases to <50% or if there is less than a twofold increase in XTT formazan production above background in control cells. Even when there is a quality control problem, if available data clearly indicate that a drug is not effective, negative results are reported. An assay is also considered to be negative if microscopic observations appear to indicate drug-induced antiviral protection by XTT formazan production but fail to show the presence of intact cells in two assays. Negative assays that pass all quality control requirements are not routinely repeated. Assays are discarded if the positive control, AZT, which is included in at least one assay in a series, fails to show the expected degree of protection; all drug assays with questionable data on the AZT control are repeated.

Results

Initial Studies

Basic protocols were established for infection of target cells and drug treatment (fig. 2). Preliminary comparisons between cocultivation (cell-to-cell) (fig. 2A) and cell-free virus (fig. 2B) modes of infection showed similar levels of protection by AZT in MT-2 or CEM-SS cells (data not shown). Therefore, a majority of the initial feasibility studies used HIV-1 (III_b) in the convenient cocultivation assay protocol with chronically infected H9 cells as the virus source.

Figures 3A and 3B illustrate the relationships among XTT formazan production, synthesis of infectious virus, and cell viability in a 7-day cocultivation assay. In this typical experiment, 10^4 CEM-SS target cells mixed with 400 infected or uninfected H9 cells were added in triplicate to wells of individual microtiter plates and assayed daily for XTT formazan production and numbers of viable cells (trypan dye exclusion) as described. Supernatant samples (50 μL) were taken daily from infected cultures, frozen in vapor-phase N_2, and subsequently tested as a group by syncytium assay for infectious virus. XTT formazan production began to plateau 4 days after the start of culture (fig. 3A). However, the total number of viable cells per milliliter in uninfected cultures, as determined by trypan blue dye exclusion (fig. 3B), continued to rise through day 7 of the culture despite a small decrease in the percentage of cell viability. The data suggest complex relationships among XTT formazan synthesis, cell viability, and virus synthesis in infected cell cultures. There were no evident differences in XTT formazan production in infected and uninfected cultures at the start of the incubation period (fig. 3A). XTT formazan synthesis in infected cultures appeared to be similar to that in uninfected controls from day 1 to about day 4, but on day 5 of culture, synthesis in the infected cultures decreased to approximately 20% of that in the uninfected controls.

The production of extracellular virus (fig. 3A) paralleled XTT formazan synthesis in infected cultures, while the number of viable cells per milliliter (fig. 3B) remained fairly steady through the 7-day period, rising to $<5 \times 10^4$ cells per well by day 5, compared with 135×10^3 cells per well in the uninfected controls. The percent of viable cells in infected cultures decreased to ≈50% by day 6. There appeared to be a continual increase in the synthesis of infectious virions (fig. 3A) that was independent of an apparent decrease in the number of viable host cells as measured by trypan blue dye exclusion. A possible basis for these apparent contradictions was revealed by microscopic observations of the cultures. Beginning 2 days after the start of culture, there was a gradual increase in the number of virus-induced multinucleated giant cells (syncytia), which resulted in a net decrease in the number of single, viable cells over time (data not shown). It is conceivable that, initially, the formation of syncytia caused an apparent decrease in cell viability (determined by trypan blue dye exclusion) without actually impacting virus synthesis. However, with time, HIV-1-induced cytopathic effects eventually result in the destruction of syncytia, suppression of XTT formazan production, and virion synthesis.

Data derived from these initial experiments also indicated that background absorbance levels in cell-free, reagent controls were unacceptably high (optical density, 0.3-0.4; data not shown). Subsequent analysis suggested that a common component of media, the pH indicator phenol red, might

be responsible for the high background optical densities observed. Thus, for all subsequent experimental work described here, phenol red was deleted from the culture medium.

Determination of Antiviral Activity

A typical test plate is illustrated in figure 4; in the plate shown, the CEM-SS cell line (*10*) is used as the target. In this example, the prototypical anti-HIV-1 agents 2′,3′-dideoxyadenosine (wells B11, C11-B4, and C4) and AZT (wells D11, E11-D4, and E4) supported cell survival and thus significant XTT formazan production over a broad range of drug concentrations. The effects of various drug dilutions on uninfected target cells are also shown in figure 4; there is a single well for each drug dilution to demonstrate potential cytotoxicity (F11-F4 for 2′,3′-dideoxyadenosine and G11-G4 for AZT).

Quantitation and Validation of Results

The quantitative data for AZT that were collected and plotted by computer are shown in figure 5. Drug comparisons are made on the basis of EC_{50} (protection in infected cells; fig. 5, bottom curve); IC_{50} (cytotoxicity in uninfected cells; fig. 5, top curve); and in vitro "therapeutic indices." In the example shown in figure 5, AZT has an EC_{50} of 0.186 μM, an IC_{50} of 191 μM, and an in vitro therapeutic index (IC_{50}/EC_{50}) of 1,027.

The effects of AZT concentration on the relationships among XTT formazan production, HIV-1 p24 antigen synthesis, and infectious virus production [syncytium assay (*10*)] are shown in figure 6. As a result of increased cellular survival, there was a dose-related increase in XTT formazan production with increasing AZT concentrations. This was followed by reduction in XTT formazan production at the highest concentrations of AZT, as a result of drug cytotoxic-

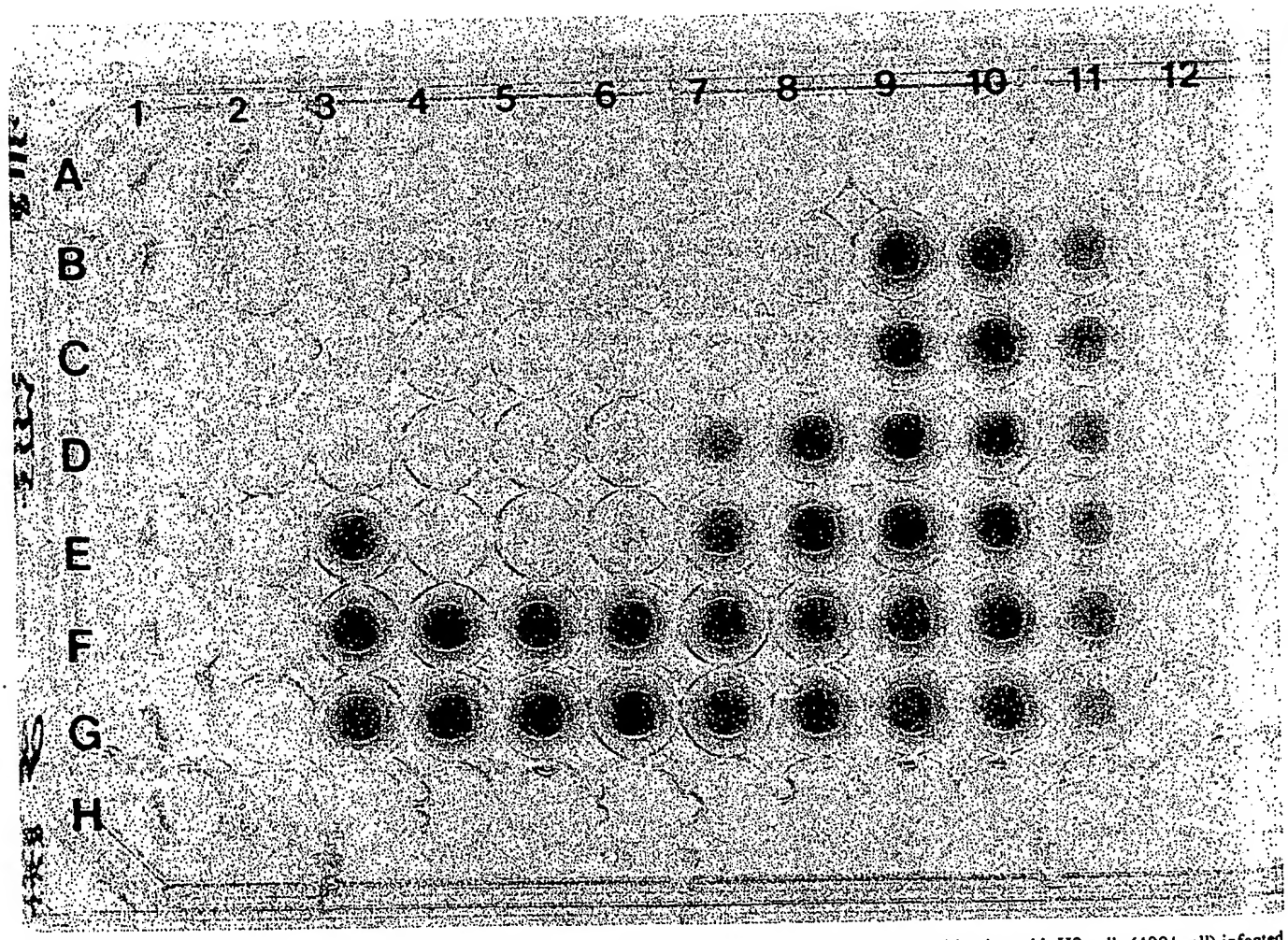

Figure 4. Typical test plate at time of assay end point. In this 7-day assay, CEM-SS cells (10^4) infected by cocultivation with H9 cells (400/well) infected with HIV-1 (III_b) were tested against AZT and 2′,3′-dideoxyadenosine (DDA). Medium free of phenol red was used throughout procedures to minimize background optical density. B2-G2: reagent control (XTT-PMS in media). B3-D3: untreated, infected control cells. E3-G3: untreated, uninfected control cells. B11, C11-B4, and C4: duplicate 10-fold dilutions of DDA (starting at 500 μM) against infected CEM-SS target cells. D11, E11-D4, and E4: duplicate 10-fold dilutions of AZT (starting at 1 mM) against infected CEM-SS target cells. F11-F4: single-well dilutions of DDA directed against uninfected, control CEM-SS cells. G11-G4: single-well dilutions of AZT directed against uninfected control CEM-SS cells. A11-A4: single-well dilutions of DDA without cells. H11-H4: single-well dilutions of AZT without cells. Orange color indicates formazan product of XTT reduction by viable cells.

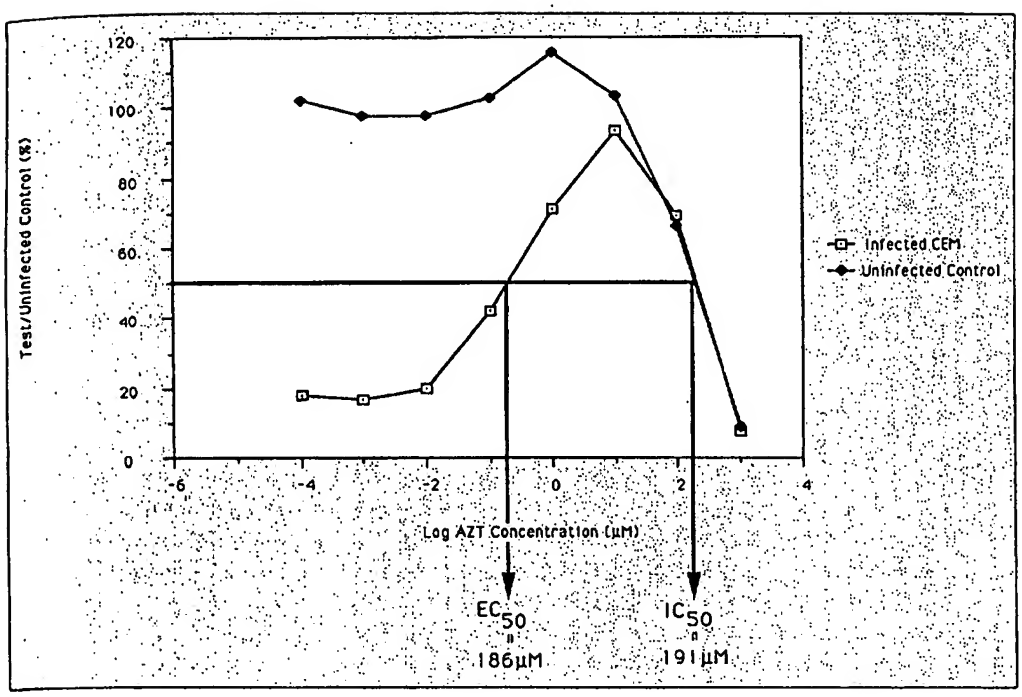

Figure 5. Quantitation of XTT formazan production in AZT-treated cultures of CEM-SS cells. Infected or uninfected CEM-SS target cells (10^4/well) treated with 10-fold, serial dilutions of AZT. EC_{50} represents concentration of AZT (e.g., 0.186 μM) that increases (protects) XTT formazan production in infected cultures to 50% of that in untreated, uninfected control cells. IC_{50} represents inhibitory or toxic concentration of AZT (e.g., 191 μM) that reduces XTT formazan production in uninfected cultures to 50% of that in untreated, uninfected control cells. Levels of XTT formazan in untreated, infected control cells were 20% of those in untreated, uninfected control cells.

ity. Cell viability, as determined by trypan blue dye exclusion, paralleled the changes in XTT formazan production (data not shown). At the same time, p24 synthesis and infectious virus production (SFU) were suppressed by AZT, relative to synthesis and production in untreated, infected control cells. This suppression was also dose dependent. Similar quantitation of drug cytotoxicity is not readily obtainable by p24 antigen analysis, and syncytium assay gives only semiquantitative results.

Effects on Cell Lines

To date, the drug screen has been conducted predominantly with two cell lines, CEM-SS (*10*) and MT-2 (*6*). Selected antiviral compounds were tested in two additional T-lymphoblastoid cell lines, C3-44 and LDV-7 (table 1). The assays in each cell line were optimized for killing of target cells by HIV-1-infected H9 cells in preliminary titration experiments. The number of cocultivated, infected H9 cells was

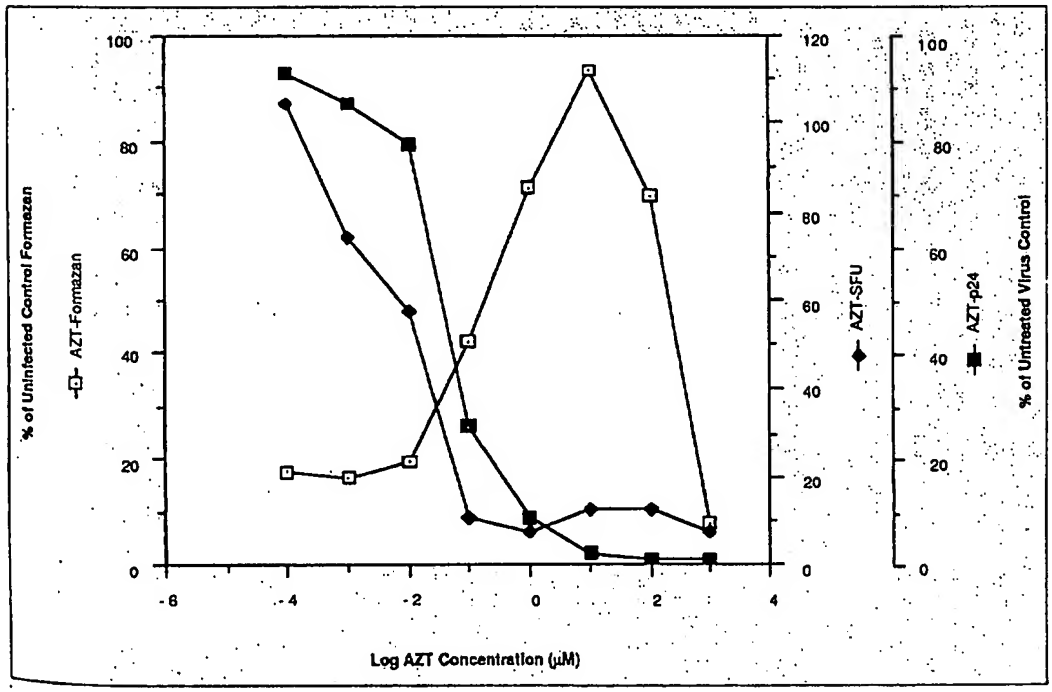

Figure 6. Correlation of XTT formazan production by infected, AZT-treated CEM-SS target cells with production of infectious virus as measured by syncytium assay and p24 antigen synthesis as determined by p24 antigen-capture ELISA (*13*).

Table 1. Comparison of T-lymphoblastoid cell line sensitivities with protection induced by various antiviral agents as determined by XTT formazan production in cocultivation protocol

Drug*	Cell (EC_{50} in μM)†			
	CEM-SS	MT-2	LDV-7	C3-44
AZT	0.2	0.012	0.05	0.007
DDA	7	1	1	2
Cyclosporine	13	6	>167	25
Pepstatin	380	>728	>728	618
Castanospermine	201	>1,100	1,015	123
ATA	35	16	57	14
Suramin analogue	28	29	41	17

*DDA = 2′,3′-dideoxyadenosine, ATA = aurintricarboxylic acid.
†EC_{50} = concentration of drug at which infected cells produce level of XTT formazan 50% of that produced by uninfected control cells.

determined so that, in each target cell culture, the XTT formazan synthesis in 7 days would be reduced at least 70%. The test drugs were selected because of their demonstrated anti-HIV-1 activities in other test systems and for known or apparent differences in their mechanisms of action. It is clear from these data that not all of the T-lymphoblastoid host cells potentially usable in the in vitro assay system are uniformly protected by known antiviral compounds.

Cell-Free Virus and Cocultivation Protocols

Although a majority of our initial feasibility studies for the XTT-based anti-HIV-1 assay were conducted with the cocultivation protocol, the daily use of this assay in a high-flux screen would require an unattainably high degree of consistency in the maintenance of both target cells and chronically infected H9 cells. A constant level of infectious virus production from the chronically infected H9 cells would be mandatory to ensure a consistent MOI input on a day-to-day basis. This requirement would be essential for assay standardization; it would provide for uniform killing of infected controls and consistency of drug-induced protection from experiment to experiment. Although the cocultivation protocol is relatively convenient and seemed otherwise potentially attractive for use in a high-flux screen, we were unable to obtain the necessary day-to-day consistency and assay standardization using this mode of virus infection. Moreover, it was not possible to precisely quantitate MOI with the cocultivation assay. The percentage of virus-producing cells appeared to fluctuate daily, and levels of virus-induced killing varied, sometimes by as much as 100%–200%. In addition, other laboratories (*18*) have recently reported marked differences between cocultivation and cell-free virus assays in the levels of protection induced by individual compounds and in the ability of a given assay to identify a protective agent.

The III_b variant of HIV-1, which we used initially in the cell-free virus protocol, produced infectious virus titers of 10^3–10^4 SFU/mL from chronically infected H9 cells. Scale-up from small feasibility studies to large-scale screening with the cell-free virus protocol (fig. 2B) was not practical with these virus preparations. On the other hand, H9 cells chronically infected with the RF variant of HIV-1 consistently produced high infectious virus titers ($>10^5$ SFU/mL). MOIs necessary to provide a consistent 70%–80% reduction of XTT formazan synthesis in the 7-day cell-free virus assay were easily established in preliminary titration experiments by use of the RF variant with MT-2 (MOI, 0.005) and CEM-SS (MOI, 0.05) target cells. Large volumes of these virus preparations have been divided into aliquots and frozen; these preparations are being used in scale-up operations in the high-flux screen with use of the cell-free virus protocol (fig. 2B). They were also used in the experiments comparing results of the cell-free virus and cocultivation protocols using MT-2 and CEM-SS target cells (table 2), which are described later in this report; these results further highlight the advantages of the cell-free virus protocol over the cocultivation protocol.

Initially, we used 10^4 target host cells per microtiter well in the cocultivation protocol (fig. 2A). This culture condition may contribute to the plateau of XTT formazan production and premature termination of infectious virus synthesis in the absence of drug treatment (fig. 3A). Because this approach weighs the assay heavily in favor of apparent drug-induced protection, it may generate unnecessarily large numbers of false-positive results. In preliminary cell-free virus studies (data not shown), it was determined that a lower number of host cells (5×10^3 per well) would permit a continued increase in XTT formazan production and infectious virus synthesis over the 7-day culture period. Thus we used this number of host target cells per well in all subsequent cocultivation and cell-free virus assays.

As we have noted, initial evaluations suggested no signif-

Table 2. Comparison of EC_{50} and IC_{50} in cell-free virus and cocultivation protocols in MT-2 and CEM-SS cells*

Drug	MT-2 cells				CEM-SS cells			
	EC_{50} (μM)		IC_{50} (μM)		EC_{50} (μM)		IC_{50} (μM)	
	CFV	Cocultivation	CFV	Cocultivation	CFV	Cocultivation	CFV	Cocultivation
AZT	0.43	0.24	>1,000	>1,000	0.05	0.65	>1,000	>1,000
DDC	2.68	NO	87.20	3.23	0.33	0.26	144.00	30.30
DDA	0.00	1.64	>500	>500	0.00	13.90	>500	>500
DDI	20.20	15.10	>625	>625	3.82	10.20	>625	>625
Dextran sulfate	0.12	5.34	>39.5	>39.5	0.03	0.95	>39.5	>39.5

*HIV-1 (RF) was used for all cell-free virus (CFV) assays. MOI was 0.005 for MT-2 cells and 0.05 for CEM-SS cells. Cocultivation protocol used 10^2 H9 cells chronically infected with HIV-1 (III_b) to infect 10^4 MT-2 target cells; 400 infected H9 cells were used to infect 10^4 CEM-SS target cells. DDC = 2′,3′-dideoxycytidine; DDA = 2′,3′-dideoxyadenosine; DDI = 2′,3′-dideoxyinosine; NO = not obtainable.

icant differences in the sensitivities of the two assay procedures to drug-induced, antiviral protection. These initial studies were conducted with a limited number of nucleoside analogues in MT-2 cells. The data in table 2 suggest that, depending on the cell line and the compound tested, the method of testing could have a significant impact on assay results. The cell-free virus assay appears to provide a more sensitive system for identifying protective compounds. These results appear to be independent of drug mechanisms, as can be seen from a comparison of the effects of two of the drugs used with the two assay protocols: 2',3'-dideoxyadenosine, a nucleoside analogue (a reverse transcriptase inhibitor), and dextran sulfate, which is purported to function at the level of virus attachment (*19,20*). Comparison of the EC_{50} values in table 2 shows that the cell-free virus assay indicates much more potent protection by dextran sulfate than that indicated by the cocultivation procedure, depending on the cell line used.

We observed differences between the cytotoxic effects of the test compounds on MT-2 and CEM-SS cell lines. The agent 2',3'-dideoxycytidine appeared to be more toxic in MT-2 cells than in CEM-SS cells. Although our data suggest that differences exist in the sensitivities of cell lines to both the protective (tables 1 and 2) and direct cytotoxic (table 2) effects of test agents, the CEM-SS line has proven overall to be the most useful host cell line for identification of potential active compounds in the primary screen. Compounds that demonstrated antiviral activity in other cell lines have seldom had negative results in the CEM-SS cell line.

Discussion

On the basis of the studies described here, we have implemented, as our high-flux primary anti-HIV-1 screen, the cell-free virus protocol (fig. 2B) using the HIV-1 (RF) virus with the CEM-SS host cell. If a test substance shows at least 30% protection against virus-induced cytopathic effects in the host cells, the microculture XTT antiviral assay is repeated. Additionally, at the termination of the assay, microscopic observations of cell survival are made, and aliquots are removed for correlative assays, such as the p24 antigen enzyme-linked immunosorbent assay (ELISA) and the syncytium assay for infectious virus.

The microculture XTT antiviral assay provides a quantitative representation of HIV-1-induced cytopathic effects as modulated by test substances. This relatively simple assay facilitates the safe and rapid determination of in vitro antiviral activity as well as direct cytotoxicity. We confirmed that this system measures infection and subsequent viral replication in target cells; we correlated XTT formazan production with the accumulation of extracellular virions, as measured by quantitative HIV-1-induced syncytium formation and p24 antigen synthesis. Our studies provided evidence for the validity of this assay. We performed double-blind studies in which AZT, an agent known to be clinically active, and other new prototype agents were reliably detected. Other potential target cell types, including both cultured and primary cells, such as peripheral blood lymphocytes and monocytes or macrophages, are being evaluated for secondary assays to further characterize antiviral protection.

While the microculture XTT antiviral assay appears to be exceedingly useful for its intended application, there are nevertheless potential technical and theoretical limitations. For example, false-positive results may be generated by occurrence of the following problems individually or in combination: (*a*) nonspecific reduction of XTT by test compounds, (*b*) poorly understood drug-cell-XTT interactions, and (*c*) human error. Though it is a rare event, restoration of XTT formazan production (protection against viral cytopathic effects) without suppression of virus replication has also been observed in our laboratory. Thus, data generated by the XTT formazan-based drug screen may occasionally be subject to misinterpretation in the absence of confirmatory analyses. Useful correlative measures for confirmation of positive results in the primary screen are provided by cell-free drug controls, microscopic observation of all samples with positive results, and secondary tests (e.g., p24 or syncytium analysis) to validate suppression of virus replication.

Other laboratories (*21,22*) have recently reported the development of chromogen or cell viability dye-based assays and have proposed their use in screening for anti-HIV drugs. However, both of these assay systems require additional centrifugation or pipetting steps at the time of end-point determination. The study by Montefiore et al. (*21*), for example, requires suspension of drug-treated, virus-infected cells in microtiter trays and transfer of this suspension to a poly-L-lysine-treated microtiter tray, followed by incubation with neutral red, washing and centrifugation to remove excess dye, and extraction of the dye into acidified ethanol prior to colorimetric quantitation. An alternative microtiter-based MTT assay was recently suggested by Pauwels et al. (*22*); this assay also uses MT-2 cells as targets. The assay is also a potentially rapid screening method, but unlike the XTT system, it requires solubilization of the resulting formazan. Thus, both the MTT and neutral red assays necessitate additional centrifugation, pipetting, or aspiration steps with all of the attendant increased potential for aerosolization and exposure to HIV.

During 1988, the microculture XTT antiviral assay system was applied as a primary screen to the evaluation of >20,000 samples (10,000 plates), with use of both CEM-SS and MT-2 target cells for each test. The 20,000 samples included ≈7,000 unique compounds; 4,000 were pure synthetic or naturally occurring compounds, and 3,000 were crude extracts or partially purified fractions from natural products. A recent report by Vince et al. (*23*) described the first new synthetic anti-HIV-1 drug candidate identified by this screen; it is a novel carbocyclic nucleoside analogue. Moreover, since the pilot-scale implementation of the screen 1 year ago, ≈70 additional compounds (≈1% of unique samples screened) have shown in vitro anti-HIV-1 activity. Although a majority of the new active compounds are analogues of known or closely related classes of antiviral agents, a few entirely new nonnucleoside classes of antiviral compounds, including synthetic as well as natural products, have been identified. For example, Gustafson et al. (manuscript submitted for publication) have described a novel series of active anti-HIV sulfolipids

discovered by XTT anti-HIV bioassay-guided isolation from *Cyanobacteria* (blue-green algae). With the full implementation of the NCI anti-HIV-1 screening program, which is anticipated by mid-1989, we expect to achieve a screening capacity of at least 40,000 samples per year. The NCI high-flux primary anti-HIV-1 screen should therefore provide a powerful new drug-discovery support resource for AIDS-targeted efforts not only intramurally within NIH, but also, as intended, extramurally at national and international levels.

In conclusion, the microculture XTT antiviral assay provides an immediate solution to the need for large-scale primary screening facilities for potential antiviral compounds. The NCI continues to investigate suitable secondary screening systems as well as alternative new primary assays to operate in parallel with or even to replace this assay. It is hoped that the availability of this national resource will facilitate the prompt identification and development of new, clinically useful anti-HIV-1 drugs.

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